Dako

HER2 FISH pharmDx™ Kit

Code K5331

6th edition

HER2 FISH pharmDx™ Kit is a direct fluorescence in situ hybridization (FISH) assay designed to quantitatively determine *HER2* gene amplification in formalin-fixed, paraffin-embedded (FFPE) breast cancer tissue specimens and FFPE specimens from patients with metastatic gastric or gastroesophageal junction adenocarcinoma.

HER2 FISH pharmDx™ Kit is indicated as an aid in the assessment of patients for whom Herceptin® (trastuzumab) treatment is being considered (see Herceptin® package insert).

The kit contains reagents sufficient for 20 tests.

Contents

Intended Use

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For breast cancer patients, results from the *HER2* FISH pharmDx™ Kit are intended for use as an adjunct to the clinicopathologic information currently used for estimating prognosis in stage II, node-positive breast cancer patients.

NOTE for gastric cancer only: All of the patients in the phase III BO18255 (ToGA) study sponsored by Hoffmann-La Roche were selected using Dako HercepTest™ (IHC) and Dako *HER2* FISH pharmDx™ Kit (FISH). However, enrollment in the BO18255 study was limited to patients whose tumors were HER2 protein overexpressing (IHC 3+) or gene amplified (FISH+; *HER2*/CEN-17 ratio ≥ 2.0). No patients were enrolled whose tumors were not gene amplified but HER2 protein weakly to strongly overexpressing [FISH(-)/IHC 2+], therefore it is unclear if patients whose tumors are not gene amplified but HER2 protein-overexpressing [i.e., FISH(-), IHC 2+ or 3+] will benefit from Herceptin® treatment. The study also demonstrated that gene amplification (FISH) and protein overexpression (IHC) are not as correlated as with breast cancer, therefore a single method should not be used to determine HER2 status.

Gastric or gastroesophageal junction adenocarcinoma is also referred to as gastric cancer in this document.

For breast cancer application, please refer to pages 5-26

For gastric cancer application, please refer to pages 27-48

Important: Please note differences for breast cancer tissue and gastric cancer tissue especially in the Interpretation of Staining Sections.

Summary and Explanation - Breast

The human *HER2* gene (also known as *ERBB2* or *NEU*) is located on chromosome 17 and encodes the HER2 protein or $p185^{HER2}$. The HER2 protein is a membrane receptor tyrosine kinase with homology to the epidermal growth factor receptor (EGFR or HER1) (1-2). The *HER2* gene is present in 2 copies in all normal diploid cells.

In a fraction of patients with breast cancer, the *HER2* gene is amplified as part of the process of malignant transformation and tumor progression (3-8). *HER2* gene amplification generally leads to overexpression of the HER2 protein on the surface of breast cancer cells (9).

Amplification of the *HER2* gene and/or overexpression of its protein have been demonstrated in 25-30% of breast cancers. This up-regulation is associated with poor prognosis, increased risk of recurrence, and shortened survival. Several studies have shown that HER2 status correlates with sensitivity or resistance to certain chemotherapy regimens (10).

Demonstration of high HER2 protein overexpression or *HER2* gene amplification is essential for initiating therapy with Herceptin®, a monoclonal antibody to HER2 protein. Clinical studies have shown that patients whose tumors have high HER2 protein overexpression and/or amplification of the *HER2* gene benefit most from Herceptin® (11).

Principle of Procedure - Breast

HER2 FISH pharmDx™ Kit contains all reagents required to complete a FISH procedure for formalin-fixed, paraffin-embedded tissue section specimens.

After deparaffinization and rehydration, specimens are heated in Pre-Treatment Solution for 10 minutes. The next step involves a proteolytic digestion using ready-to-use Pepsin at room temperature for 5-15 minutes or at 37 °C for 3 minutes. Following the heating and proteolytic pre-treatment steps, this kit employs a ready-to-use FISH Probe Mix based on a combination of PNA (peptide nucleic acid) (12) and DNA technology. This Probe Mix consists of a mixture of Texas Red-labelled DNA probes covering a 218 kb region including the *HER2* gene on chromosome 17, and a mixture of fluorescein-labelled PNA probes targeted at the centromeric region of chromosome 17 (CEN-17). The specific hybridization to the two targets results in formation of a distinct red fluorescent signal at each *HER2* gene locus and a distinct green fluorescent signal at each chromosome 17 centromere. To diminish background staining, the Probe Mix also contains unlabelled PNA blocking probes. After a stringent wash, the specimens are mounted with Fluorescence Mounting Medium containing DAPI and coverslipped. Using a fluorescence microscope equipped with appropriate filters (see Appendix 3), tumor cells are located, and enumeration of the red *(HER2)* and green (CEN-17) signals is conducted. Then the *HER2*/CEN-17 ratio is calculated. Normal cells in the analyzed tissue section will serve as an internal positive control of pre-treatment and hybridization efficiency.

For details see the Interpretation of Staining section.

For interactive e-learning please use the *HER2* FISH pharmDx™ e-learning program designed to supply laboratory technicians, pathologists and scientists with an accurate and fast knowledge of how to achieve optimal results using *HER2* FISH pharmDx™ Kit: www.dako.com

Reagents - Breast

Materials provided
The materials listed below are sufficient for 20 tests (a test is defined as one 22 mm x 22 mm target area). The number of tests is based on the use of 5-8 drops (250 µL per slide of Vial 2, 10 µL per slide of Vial 3, and 15 µL per slide of Vial 5). The kit provides materials sufficient for 10 individual staining runs.

HER2 FISH pharmDx™ Kit is shipped on dry ice. **To ensure that kit components have not been exposed to high temperatures during transport, dry ice should still be present upon receipt**. Note that some kit components may remain unfrozen, this will not affect the performance of the *HER2* FISH pharmDx™ Kit.

Vial 1

PRE-TREATMENT SOLUTION (20x)

Pre-Treatment Solution (20x)
150 mL, concentrated 20x
MES (2-[*N*-morpholino]ethanesulphonic acid) buffer.

Vial 2

PEPSIN

Pepsin
7.5 mL, ready-to-use
Pepsin solution, pH 2.0; contains stabilizer and an antimicrobial agent.

Vial 3

HER2/CEN-17 PROBE MIX

***HER2*/CEN-17 Probe Mix**
0.2 mL, ready-to-use
Mix of Texas Red-labelled *HER2* DNA probes and fluorescein-labelled CEN-17 PNA probes; supplied in hybridization buffer with 45% formamide, stabilizer, and unlabelled PNA blocking probes.

Vial 4

STRINGENT WASH BUFFER (20x)

Stringent Wash Buffer (20x)
150 mL, concentrated 20x
SSC (saline-sodium citrate) buffer with detergent.

Vial 5

FLUORESCENCE MOUNTING MEDIUM

Fluorescence Mounting Medium
0.3 mL, ready-to-use
Fluorescence mounting medium with 100 µg/L DAPI (4',6-diamidine-2-phenylindole).

Vial 6

WASH BUFFER (20x)

Wash Buffer (20x)
500 mL, concentrated 20x
Tris/HCl buffer.

COVERSLIP SEALANT

Coverslip Sealant
1 tube, ready-to-use
Solution for removable sealing of coverslips.

NOTE: All reagents, including Pre-Treatment Solution, Stringent Wash Buffer, and Fluorescence Mounting Medium, are formulated specifically for use with this kit.

Materials required but not provided

Laboratory reagents

Distilled or deionized water

Ethanol, 96%

Xylene or xylene substitutes

Laboratory equipment

Absorbent wipes

Adjustable pipettes

Calibrated partial immersion thermometer (range 37-100 □C)

Calibrated surface thermometer (range 37-100 □C)

Coverslips (22 mm x 22 mm)

Forceps

Fume hood

Dako Hybridizer (Code S2450)*

Heating block or hybridization oven for denaturation (82 (±2) °C)*

Humid hybridization chamber*

Slides, Dako Silanized Slides, Code S3003, or poly-L-lysine-coated slides (see Specimen Preparation)

Staining jars or baths

Timer (capable of 2-15 minute intervals)

Water bath with lid (capable of maintaining 65 (±2) °C to 99 °C)

Microwave oven with sensing capability if pre-treatment is performed using microwave oven (see B3. Staining protocol. Step 1: Pre-treatment, Method B)

* Heating block or hybridization oven for denaturation (82 (±2) °C) and hybridization (45 (±2) °C) together with a humid hybridization chamber can be used as an alternative to Dako Hybridizer.

Microscope equipment and accessories

Filters for fluorescence microscope: DAPI and FITC/Texas Red double filter, or FITC and Texas Red mono filters - see Appendix 3 for details

Fluorescence microscope with a 100 watt mercury lamp is recommended

Microscope slide folder (cardboard tray for 20 slides with hinged cover or similar)

Precautions - Breast

1. For in vitro diagnostic use.
2. For professional users.
3. Vial 1, Pre-Treatment Solution (20x), contains 1-<20% 2-morpholinoethanesulphonic acid; Vial 2, Pepsin, contains 5-10% propan-2-ol; Vial 4, Stringent Wash Buffer (20x), contains 1-<5% octoxinol; and Vial 6, Wash Buffer (20x), contains 1-<20% trometamol. At product concentrations these substances do not require hazard labelling. Material Safety Data Sheets (MSDSs) are available for professional users on request.
4. Vial 2, Pepsin, contains pepsin A that may cause an allergic reaction.
5. Vial 3, *HER2*/CEN-17 Probe Mix contains 45% formamide and is labelled:
   Toxic.
   R61 May cause harm to the unborn child.
   S45 In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).
   S53 Avoid exposure – obtain special instructions before use.
   S60 This material and/or its container must be disposed of as hazardous waste.
   As a general rule, persons under 18 years of age are not allowed to work with this product. Users must be carefully instructed in the proper working procedure, the dangerous properties of the product and the necessary safety instructions. Please refer to the Material Safety Data Sheet (MSDS) for additional information.
6. Coverslip Sealant contains 60-100% naphtha (petroleum), hydrotreated light, and is labelled:
   Extremely flammable.
   Dangerous for the environment.
   R11 Highly flammable.
   R51/53 Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.
   S9 Keep container in a well-ventilated place.
   S16 Keep away from sources of ignition – No smoking.
   S35 This material and its container must be disposed of in a safe way.
   S57 Use appropriate container to avoid environmental contamination.
   S61 Avoid release to the environment. Refer to special instructions/safety data sheets.
   Please refer to the Material Safety Data Sheet (MSDS) for additional information.
7. Specimens, before and after fixation, and all materials exposed to them, should be handled as if capable of transmitting infection and should be disposed of with proper precautions (13). Never pipette reagents by mouth and avoid contacting the skin and mucous membranes with reagents and specimens. If reagents come in contact with sensitive areas, wash with copious amounts of water.
8. Minimize microbial contamination of reagents to avoid erroneous results.
9. Incubation times and temperatures, or methods other than those specified, may give erroneous results.
10. Tissue fixation methods and thickness of specimen other than those specified may affect tissue morphology and/or signal intensity.
11. Avoid evaporation of *HER2*/CEN-17 Probe Mix during hybridization by ensuring sufficient humidity in the hybridization chamber.
12. Reagents have been optimally diluted. Further dilution may result in loss of performance.
13. Wear appropriate personal protective equipment to avoid contact with eyes and skin. Please refer to the Material Safety Data Sheet (MSDS) for additional information.

Storage - Breast

Store in the dark at 2-8 °C. All reagents tolerate frozen storage. Freezing and thawing the reagents for each analysis does not affect performance.

The Pepsin, *HER2*/CEN-17 Probe Mix, and Fluorescence Mounting Medium (Vials 2, 3 and 5) may be affected adversely if exposed to heat. Do not leave these components at room temperature. The *HER2*/CEN-17 Probe Mix and Fluorescence Mounting Medium (Vials 3 and 5) may be affected adversely if exposed to excessive light levels. Do not store these components or perform analysis in strong light, such as direct sunlight.

Do not use the kit after the expiration date stamped on the kit box. If reagents are stored under conditions other than those specified in this package insert, the user must validate reagent performance (14).

There are no obvious signs to indicate instability of this product. Therefore, it is important to evaluate normal cells in the analyzed tissue section. If an unexpected fluorescence pattern is observed which cannot be explained by variations in laboratory procedures, and a problem with the *HER2* FISH pharmDx™ Kit is suspected, contact our Technical Services.

Specimen Preparation - Breast

Specimens from biopsies, excisions or resections must be handled to preserve the tissue for FISH analysis. Standard methods of tissue processing for immunocytochemical staining should be used for all specimens (15).

Paraffin-embedded sections

Only tissue preserved in neutral buffered formalin and paraffin-embedded is suitable for use. Specimens should e.g. be blocked into a thickness of 3 or 4 mm and fixed for 18-24 hours in neutral buffered formalin. The tissues are then dehydrated in a graded series of ethanol and xylene, followed by infiltration by melted paraffin held at no more than 60 °C. Properly fixed and embedded tissues will keep indefinitely prior to sectioning and slide mounting if stored in a cool place (15-25 °C) (15-16). Other fixatives are not suitable.

Tissue specimens should be cut into sections of 4-6 µm.

The slides required for *HER2* gene amplification analysis and verification of tumor presence should be prepared at the same time. A minimum of 2 serial sections is recommended, 1 section for tumor presence stained with hematoxylin and eosin (H&E stain), and 1 section for *HER2* gene amplification analysis. It is recommended that tissue sections are mounted on Dako Silanized Slides, Code S3003, or poly-L-lysine-coated slides. Specimens should be analyzed within 4-6 months of sectioning when stored at room temperature (20-25 °C).

INSTRUCTIONS FOR USE - Breast

A. Reagent Preparation - Breast

It is convenient to prepare the following reagents prior to staining:

A.1 Pre-Treatment Solution

Crystals may occur in Vial 1, but they will dissolve at room temperature. Ensure that no crystals are present before preparation of reagent.

Dilute a sufficient quantity of Vial 1 (Pre-Treatment Solution 20x) by diluting the concentrate 1:20 in distilled or deionized water. Unused diluted solution may be stored at 2-8 °C for one month. Discard diluted solution if cloudy in appearance.

A.2 Stringent Wash Buffer

Dilute a sufficient quantity of Vial 4 (Stringent Wash Buffer 20x) by diluting the concentrate 1:20 in distilled or deionized water. Unused diluted buffer may be stored at 2-8 °C for one month. Discard diluted buffer if cloudy in appearance.

A.3 Wash Buffer

Dilute a sufficient quantity of Vial 6 (Wash Buffer 20x) by diluting the concentrate 1:20 in distilled or deionized water. Unused diluted buffer may be stored at 2-8 °C for one month. Discard diluted buffer if cloudy in appearance.

A.4 Ethanol series

From a 96% ethanol solution, prepare 3 jars with 70%, 85%, and 96% ethanol, respectively. Store covered jars at room temperature or at 2-8 °C, and use for a maximum of 200 slides. Discard solutions if cloudy in appearance.

B. Staining Procedure - Breast

B.1 Procedural notes

The user should read these instructions carefully and become familiar with all components prior to use (see Precautions).

If kit components are stored frozen, it is recommended to move the reagents to 2-8 °C the day before performing the analysis to allow proper temperature equilibration. All reagents should be equilibrated to the relevant temperature prior to use as follows:

Vial 1: The diluted Pre-Treatment Solution should be equilibrated to **95-99 °C** if water bath is used for pre-treatment (B3. Staining protocol, Step 1: Pre-Treatment Method A). If microwave oven with sensing capability is used for pre-treatment (B3. Staining protocol, Step 1: Pre-Treatment, Method B) the diluted Pre-Treatment Solution should be equilibrated to room temperature **20-25 °C**.

Vial 2: Pepsin should be applied **at 2-8 °C** and kept cold continuously.

Vial 3: *HER2*/CEN-17 Probe Mix may be applied at any temperature from **2-25 °C.**

Vial 4: The Diluted Stringent Wash Buffer; one jar should be equilibrated to room temperature, another jar should be equilibrated to **65 (±2) °C** prior to use.

Vial 5: Fluorescence Mounting Medium may be applied at any temperature from **2-25 °C.**

Vial 6: The Diluted Wash Buffer should be equilibrated to room temperate **20-25 °C.**

The Coverslip Sealant may be applied at any temperature from **2-25 °C.**

All steps must be performed at the outlined temperature.

The procedure includes a number of dehydrations followed by drying of the tissue sections. Ensure that tissue sections are completely dry before proceeding to the next step. Do not allow tissue sections to dry during the other procedural steps.

If the staining procedure has to be interrupted, slides may be kept in Wash Buffer after the deparaffinization step for up to 1 hour at room temperature (20-25 °C) without affecting the results.

B.2 Treatment of tissues prior to staining

Deparaffinization and rehydration: Prior to performing the analysis, tissue slides must be deparaffinized to remove embedding medium and rehydrated. Avoid incomplete removal of paraffin. Residual embedding medium will result in increased non-specific staining. This step should be performed at room temperature (20-25 °C).

1. Place slides in a xylene bath and incubate for 5 (±1) minutes. Change baths and repeat once.
2. Tap off excess liquid and place slides in 96% ethanol for 2 (±1) minutes. Change baths and repeat once.
3. Tap off excess liquid and place slides in 70% ethanol for 2 (±1) minutes. Change baths and repeat once.

4. Tap off excess liquid and place slides in diluted Wash Buffer (see INSTRUCTIONS FOR USE, Section A.3) for a minimum of 2 minutes. Commence staining procedure as outlined in Section B.3, Step 1, Pre-Treatment.

Xylene and alcohol solutions should be changed after 200 slides or less.

Xylene substitutes may be used.

NOTE: The reagents and instructions supplied in this kit have been designed for optimal performance. Further dilution of the reagents or alteration of incubation temperatures may give erroneous or discordant results. Differences in tissue processing and technical procedures in the user's laboratory may invalidate the assay results.

B.3 Staining protocol

DAY 1

Step 1: Pre-Treatment

Pre-treatment can be performed either by using water bath as described in method A) or, alternatively, by use of microwave oven with sensing capability as described in method B)

Method A) Pre-treatment using water bath:

Fill staining jars, e.g. Coplin jars, with the diluted Pre-Treatment Solution (see INSTRUCTIONS FOR USE, Section A.1). Place staining jars containing Pre-Treatment Solution in water bath. Heat water bath and the Pre-Treatment Solution to 95-99 °C. Measure temperature inside jar with a calibrated thermometer to ensure correct temperature. Cover jars with lids in order to stabilize the temperature and avoid evaporation.

Immerse the room temperature deparaffinized sections into the preheated Pre-Treatment Solution in the staining jars. Re-check temperature and incubate for 10 (±1) minutes at
95-99 °C.

Remove the entire jar with slides from the water bath. Remove lid and allow the slides to cool in the Pre-Treatment Solution for 15 minutes at room temperature.

Transfer the slides to a jar with diluted Wash Buffer (see INSTRUCTIONS FOR USE, Section A.3) for 3 minutes at room temperature (20-25 °C).

Replace Wash Buffer and soak sections for another 3 minutes.

Method B) Pre-Treatment using microwave oven with sensing capability:

Fill a plastic jar with diluted room temperature (20-25 °C) Pre-Treatment Solution. Immerse the deparaffinized sections in Pre-Treatment Solution, cover the jar with a punctured lid and place it in the microwave oven. Select the boiling sensor function and a program that runs for 10 minutes after boiling temperature has been reached*.

Following the 10 minutes incubation take the jar with slides out of the oven, remove the lid and cool for 15 minutes at room temperature. Transfer the slides to a jar with diluted Wash Buffer and soak for 3 minutes at room temperature (20-25 °C). Replace Wash Buffer and soak sections for another 3 minutes.

* The use of a microwave oven with a sensing capability means that the oven must include a sensor and programs which initially heat the Pre-Treatment Solution to the boiling point and subsequently maintain the required pre-treatment temperature (above 95 °C) while counting down the preset time (10 (±1) minutes). Some microwave oven models with sensing capability may not include the possibility to freely set a count-down time. If the model only includes pre-set programs, be sure to select a program which maintain the required pre-treatment temperature (above 95 °C) for at least 10 (±1) minutes and manually stop the program after 10 (±1) minutes.

NOTE: The Pre-Treatment Solution is designed for a single use application only. Do not re-use.

Step 2: Pepsin, ready-to-use

Pepsin incubation can be performed either at room temperature (20-25 °C) as described in method A) or, alternatively, at 37 °C as described in method B).

Tap off excess buffer. Using lintless tissue (such as an absorbent wipe or gauze pad), carefully wipe around the specimen to remove any remaining liquid and to keep reagents within the prescribed area.

Apply 5-8 drops (250 µL) of cold (2-8 °C) Pepsin (Vial 2) to cover specimen. Always store Pepsin at 2-8 °C.

Method A) Incubation at room temperature:

Incubate for 5-15 minutes at room temperature (20-25 °C). An incubation time of 10 minutes will be adequate for most specimens, but the optimal incubation time may depend on tissue fixation and/or thickness of specimen and should be determined by the user.

Tap off Pepsin and soak sections in the diluted Wash Buffer (see INSTRUCTIONS FOR USE, Section A.3) for 3 minutes at room temperature (20-25 °C).

Replace diluted Wash Buffer and soak sections for another 3 minutes. Continue to dehydration.

Method B) Incubation at 37 °C:

Place specimen with Pepsin on a heating block at 37 °C – e.g. Dako Hybridizer – and incubate for 3 minutes. An incubation time of 3 minutes will be adequate for most specimens, but the optimal incubation time may depend on tissue fixation and/or thickness of specimen and should be determined by the user.

Tap off Pepsin and soak sections in diluted Wash Buffer for 3 minutes at room temperature (20-25 °C).

Replace Wash Buffer and soak sections for another 3 minutes. Continue to dehydration.

Dehydrate tissue sections through a graded series of ethanol: 2 minutes in 70% ethanol, 2 minutes in 85% ethanol, and 2 minutes in 96% ethanol.

Allow tissue sections to air dry completely.

Step 3: *HER2*/CEN-17 Probe Mix, ready-to-use

The following step should be performed in a fume hood.

Apply 10 µL of *HER2*/CEN-17 Probe Mix (Vial 3) to the centre of the tissue section. Immediately place a 22 mm x 22 mm glass coverslip over the Probe Mix and allow it to spread evenly under the coverslip. Avoid air bubbles. If air bubbles are observed, gently tap them away from the tissue using forceps.

Seal coverslip with Coverslip Sealant by ejecting the Sealant around the periphery of the coverslip. Allow the Coverslip Sealant to overlap the coverslip and the slide, thereby forming a seal around the coverslip. Make sure that the Coverslip Sealant covers the entire edge of the coverslip.

Prepare Dako Hybridizer* (Code S2450) for a hybridization run. Make sure that Humidity Control Strips (Code S2452) are saturated and optimal for use. Start the Hybridizer and choose a program that will denature at 82 °C for 5 minutes and hybridize overnight (14-20 hours) at 45 °C (please refer to Dako Hybridizer Instruction Manual for details). Place slides in the Hybridizer, make sure the lid is properly closed and start program.

*Instrumentation that allows for conditions similar to the ones described above may be used for denaturation and hybridization:

Place slides on a flat metal or stone surface (heating block or on a block in a hybridization oven) preheated to 82 (±2) °C. Denature for 5 minutes ensuring that the temperature of the block does not drop below 80 °C at any time.

Place slides in a preheated humidified hybridization chamber. Cover the chamber with a lid and incubate overnight (14-20 hours) at **45 (±2) °C**. Please note that a hybridization temperature of 37 °C is not suitable for use with the probes contained within this kit.

DAY 2

Step 4: Stringent Wash

Fill two staining jars, e.g. Coplin jars, with the diluted Stringent Wash Buffer (see INSTRUCTIONS FOR USE, Section A.2). A minimum volume of 100 mL or 15 mL per slide in each jar is recommended.

Place one of the staining jars containing diluted Stringent Wash Buffer at room temperature in a fume hood and the other in a water bath. Heat water bath and the diluted Stringent Wash Buffer to 65 (±2) °C. Ensure that the temperature has stabilized. Cover jar with lid in order to stabilize the temperature and avoid evaporation. Measure temperature inside the water bath jar with a calibrated thermometer to ensure correct temperature. The Stringent Wash Buffer contains detergent and may become turbid at 65 °C; this will not affect performance.

Using forceps or gloves, take slides from the hybridization chamber and gently remove Coverslip Sealant as well as coverslip and place slides in the room temperature pre-wash jar, one at a time.

As soon as all coverslips have been removed, transfer slides from the room temperature, pre-wash jar to the 65 (±2) °C jar in the water bath. Perform stringent wash for exactly 10 minutes at 65 (±2) °C.

Remove slides from the diluted Stringent Wash Buffer, and soak sections in diluted Wash Buffer for 3 minutes at room temperature (20-25 °C).

Change diluted Wash Buffer and soak sections for another 3 minutes.

Dehydrate tissue sections through a graded series of ethanol: 2 minutes in 70% ethanol, 2 minutes in 85% ethanol, and 2 minutes in 96% ethanol.

Allow tissue sections to dry completely.

Step 5: Mounting

Apply 15 µL of Fluorescence Mounting Medium containing DAPI (Vial 5) to the target area of the slide and apply a glass coverslip.

NOTE: Slides may be read after 15 minutes or within 7 days after mounting. However, fading occurs if slides are exposed to light or high temperatures. To minimize fading, store slides in the dark at 2-8 °C.

Quality Control - Breast

1. Signals must be bright, distinct and easy to evaluate.
2. Normal cells allow for an internal control of the staining run.
   - Normal cells should have 1-2 clearly visible green signals indicating that the CEN-17 PNA Probe has successfully hybridized to the centromeric region of chromosome 17.
   - Normal cells should also have 1-2 clearly visible red signals indicating that the *HER2* DNA Probe has successfully hybridized to the *HER2* amplicon.
   - Due to tissue sectioning, some normal cells will have less than the expected 2 signals of each colour.
   - Failure to detect signals in normal cells indicates assay failure, and results should be considered invalid.
3. Nuclear morphology must be intact when evaluated using a DAPI filter. Numerous ghost-like cells and a general poor nuclear morphology indicate over-digestion of the specimen, resulting in loss or fragmentation of signals. Such specimens should be considered invalid.
4. Differences in tissue fixation, processing, and embedding in the user's laboratory may produce variability in results, necessitating regular evaluation of in-house controls.

Interpretation of Staining - Breast

Assessable tissue

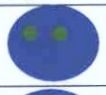
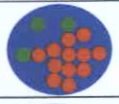
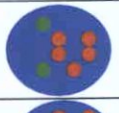
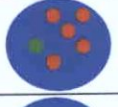
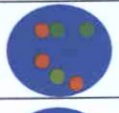
Only specimens from patients with invasive carcinoma should be tested. In cases with carcinoma in situ and invasive carcinoma in the same specimen, only the invasive component should be scored. Avoid areas of necrosis and areas where the nuclear borders are ambiguous. Do not include nuclei that require subjective judgement. Skip nuclei with weak signal intensity and non-specific or high background.

Signal enumeration: Locate the tumor within the context of the H&E stained slide and evaluate the same area on the FISH stained slide. Scan several areas of tumor cells to account for possible heterogeneity. Select an area having good nuclei distribution. Begin analysis in the upper left quadrant of the selected area and, scanning from left to right, count the number of signals within the nuclear boundary of each evaluated nucleus according to the guidelines below (see also Appendix 3).

- Focus up and down to find all of the signals in the individual nucleus.
- Count two signals that are the same size and separated by a distance equal to or less than the diameter of the signal as only one signal.
- In nuclei with high levels of *HER2* gene amplification, the *HER2* signals may be positioned very close to each other forming a cluster of signals. In these cases the number of *HER2* signals cannot be counted, but must be estimated. Special attention must be paid to the green signals, as clusters of *HER2* signals can cover the green signals making them impossible to see. In case of doubt, please check the green signals using a specific FITC filter.

Do not score nuclei without signals or with signals of only one colour. Score only those nuclei with one or more FISH signals of each colour.

Signal counting guide

1		Do not count. Nuclei are overlapping, not all areas of nuclei are visible
2		Two green signals, do not score nuclei with signals of only one color
3		Count as 3 green and 12 red signals (cluster estimation)
4		Count as 1 green and 1 red signal. Two signals of the same size and separated by a distance equal to or less than the diameter of one signal are counted as one
5		Do not count (over- or underdigested nuclei). Missing signals in the centre of nuclei (donut-shaped nuclei).
6		Count as 2 green and 3 red signals. Two signals of the same size and separated by a distance equal to or less than the diameter of one signal are counted as one
7		Count as 1 green and 5 red signals
8		Count as 3 green (1 green out of focus) and 3 red signals
9		Cluster of red signals hiding green signals, check the green signals with a specific FITC filter, or do not count

Record counts in a table as shown in Appendix 2.

Count 20 nuclei per tissue specimen, when possible from distinct tumor areas (17).

Calculate the *HER2*/CEN-17 ratio by dividing the total number of red *HER2* signals by the total number of green CEN-17 signals.

Specimens with a *HER2*/CEN-17 ratio above or equal to 2 should be considered *HER2* gene amplified (3, 17-19).

Results at or near the cut-off (1.8-2.2) should be interpreted with caution.

If the ratio is borderline (1.8-2.2), count an additional 20 nuclei and recalculate the ratio for the 40 nuclei.

In case of doubt, the specimen slide should be re-scored. For borderline cases a consultation between the pathologist and the treating physician is warranted.

Limitations - Breast

1. FISH is a multi-step process that requires specialized training in the selection of the appropriate reagents, as well as in tissue selection, fixation, and processing, preparation of the FISH slide, and interpretation of the staining results.
2. FISH results are dependent on the handling and processing of the tissue prior to staining. Improper fixation, washing, drying, heating, sectioning, or contamination with other tissues or fluids may influence on probe hybridization. Inconsistent results may be due to variations in fixation and embedding methods, or to inherent irregularities within the tissue.
3. For optimal and reproducible results, the tissue slides must be deparaffinized completely. The paraffin removal needs to be completed at the beginning of the staining process. (See INSTRUCTIONS FOR USE, section B.2).
4. Only temperature-calibrated water bath, heating block, and hybridization oven should be used. Use of other types of equipment may result in evaporation of *HER2*/CEN-17 Probe Mix during hybridization and must be validated by user.

Performance Characteristics - Breast

Hybridization efficacy

Hybridization efficacy of the *HER2* FISH pharmDx™ Kit was investigated at a routine pathology laboratory. 126 formalin-fixed, paraffin-embedded tissue sections were tested using the recommended procedure. Out of the 126 specimens, 124 could be scored according to the product guidelines, while 2 specimens could not be scored owing to technical reasons. Thus, the hybridization efficacy was 124/126 = 98% (20).

Analytical sensitivity

The sensitivity of the *HER2*/CEN-17 Probe Mix was investigated using 1 cell line with and 2 cell lines without amplification of the *HER2* gene. The ratio between the number of *HER2* signals and CEN-17 signals was calculated based on a counting of 60 nuclei per cell line.

The amplified cell line was scored as amplified with an average ratio of 3.21, while the 2 non-amplified cell lines were scored as non-amplified with average ratios of 0.96 and 1.17.

Furthermore, the *HER2*/CEN-17 ratios of 5 *HER2* gene non-amplified tissue sections were determined with the *HER2* FISH pharmDx™ Kit. Each tissue section was scored by 3 independent technicians. Results are presented in Table 1.

Table 1. *HER2*/CEN-17 ratios in 5 non-amplified tissues scored by 3 independent technicians

	Tissue 1	Tissue 2	Tissue 3	Tissue 4	Tissue 5
Technician 1	0.95	1.01	1.04	1.21	1.15
Technician 2	1.05	1.00	0.99	1.11	1.09
Technician 3	1.05	1.00	0.92	1.14	1.06
Mean ratio	1.02	1.00	0.98	1.15	1.10
CV%	6	1	6	4	4

CV: Coefficient of variation

The study confirmed all 5 tissue sections to be non-amplified with a mean *HER2*/CEN-17 ratio close to 1.0.

Analytical specificity

The *HER2* DNA probes in the *HER2*/CEN-17 Probe Mix have been end-sequenced and mapped to confirm a total coverage of 218 kb including the *HER2* gene.

The CEN-17 PNA probes in the *HER2*/CEN-17 Probe Mix have been tested individually and in combination to confirm their specific hybridization to the centromeric region of chromosome 17.

To exclude cross-hybridization to chromosomes other than chromosome 17, studies were performed on metaphase spreads according to standard Dako QC procedures. A total of 250 metaphase spreads were evaluated for specific hybridization of the *HER2* DNA and CEN-17 PNA probe mixes. In all 250 cases the hybridization was specific for chromosome 17. No cross-hybridization to loci on other chromosomes was observed in any of the 250 cases.

Testing of normal tissue

To establish a range of results for normal tissue, a study was conducted that measured the distribution of *HER2*/CEN-17 ratios in normal breast tissue specimens. In a sample set of 21 normal breast tissue specimens, the average *HER2*/CEN-17 ratio was 1.06 with a 95% confidence interval of 1.02-1.10. The standard deviation was 0.09.

Robustness studies

The robustness of the *HER2* FISH pharmDx™ assay was tested by varying pre-treatment time and temperature, pepsin incubation time, denaturation temperature, hybridization time and temperature, and stringent wash time and temperature.

No significant difference in results was observed at the following experimental conditions:

- Pretreatment at 7, 10 and 13 minutes combined with each of the temperatures 89, 92 and 95-99 °C.
- Pepsin incubation times of 2, 5, 10, 15 and 18 minutes.
- Denaturation temperatures of 72, 77, 82, 87 and 92 °C.
- Hybridization time of 17 hours combined with each of the temperatures 40, 45 and 50 °C.
- Hybridization times of 10, 12 and 14 hours at a temperature of 45 °C.

The stringent wash was tested for 10 minutes at 60, 65 and 70 °C. Additionally, the stringent wash was tested for 5, 10 and 15 minutes at 65 °C. Stringent wash for 10 minutes at 70 °C, and stringent wash for 15 minutes at 65 °C resulted in loss of signals, whereas no significant difference in results was observed at the other time/temperature combinations. Furthermore, the following dilutions of Stringent Wash Buffer were

tested: 1:10, 1:15, 1:20, 1:30 and 1:40. The 1:40 dilution of Stringent Wash Buffer resulted in loss of signals, whereas no significant difference in signal intensity was observed at the other dilutions.

Repeatability

The repeatability of the *HER2*/CEN-17 ratio was investigated with the *HER2* FISH pharmDx™ Kit using consecutive sections of normal breast tissue and breast carcinoma. The coefficient of variation for normal breast tissue was found to be 6% and 4% for breast carcinoma.

A total of 10 consecutive sections of breast cancer tissue with different thickness (duplicates of 3, 4, 5, 6, and 7 µm) were tested with the *HER2* FISH pharmDx™ Kit. The coefficient of variation of the *HER2*/CEN-17 ratio in this study was found to be 12%, i.e. higher than for tissue sections of equal thickness.

Reproducibility

The *HER2* FISH pharmDx™ Kit was tested for lot-to-lot, day-to-day and observer-to-observer reproducibility using 3 different formalin-fixed and paraffin-embedded cell lines (the non-amplified MDA-231 and MDA-175, and the *HER2* gene amplified SKBR3). In these three studies, 30 nuclei were counted per specimen. The greatest *HER2*/CEN-17 ratio variation (8%) was found in the observer-to-observer study on the amplified cell line. This might be expected and possibly reflects certain subjectivity in signal interpretation and enumeration. Results expressed as mean ratio, standard deviation, and coefficients of variation are presented in Tables 2-4.

Table 2. Lot-to-lot reproducibility. *HER2*/CEN-17 ratio measured for 3 different lots of *HER2* FISH pharmDx™ Kit

Cell line	*HER2*/CEN-17 ratio	Kit lot 1	Kit lot 2	Kit lot 3	Total
MDA-231	Mean	1.06	1.04	1.07	1.06
	SD	0.04	0.04	0.05	0.04
	CV%	4	4	4	4
	N	5	5	5	15
MDA-175	Mean	1.23	1.20	1.16	1.20
	SD	0.02	0.05	0.07	0.06
	CV%	1	4	6	5
	N	5	5	5	15
SKBR3	Mean	3.99	3.77	3.82	3.86
	SD	0.18	0.19	0.29	0.23
	CV%	5	5	8	6
	N	5	5	5	15

SD: Standard deviation CV: Coefficient of variation N: number of slides

Table 3. Day-to-day reproducibility. *HER2*/CEN-17 ratio measured on 4 different days

Cell line	*HER2*/CEN-17 ratio	Day 1	Day 2	Day 3	Day 4	Total
MDA-231	Mean	1.04	1.03	1.05	0.99	1.03
	SD	0.05	0.02	0.03	0.01	0.04
	CV%	5	2	3	1	3
	N	5	5	5	5	20
MDA-175	Mean	1.26	1.17	1.25	1.16	1.21
	SD	0.06	0.04	0.07	0.04	0.07
	CV%	5	4	6	3	6
	N	5	5	5	5	20
SKBR3	Mean	4.30	4.59	4.56	4.09	4.39
	SD	0.39	0.32	0.15	0.08	0.32
	CV%	9	7	3	2	7
	N	5	5	5	5	20

SD: Standard deviation CV: Coefficient of variation N: Number of slides

Table 4. Observer-to-observer reproducibility. *HER2*/CEN-17 ratio measured by 3 different observers

Cell line	*HER2*/CEN-17 ratio	Obs. 1	Obs. 2	Obs. 3	Total
MDA-231	Mean	1.03	1.03	1.09	1.05
	SD	0.02	0.08	0.05	0.06
	CV%	2	8	5	6
	N	5	5	5	15
MDA-175	Mean	1.17	1.15	1.11	1.14
	SD	0.02	0.05	0.11	0.07
	CV%	2	4	10	6
	N	5	5	5	15
SKBR3	Mean	4.03	3.57	3.63	3.74
	SD	0.18	0.19	0.24	0.29
	CV	5	5	7	8
	N	5	5	5	15

SD: Standard Deviation CV: Coefficient of Variation N. Number of slides

A second interobserver study was conducted on archived breast cancer tissue specimens selected to reflect a range of *HER2* amplification levels. Three observers counted events in 20 nuclei for each of 27 specimens. Concordance between

observers with regard to amplification/non-amplification status (see Interpretation of Staining) was 100% in all cases.

Assay portability

To assess interlaboratory reproducibility (assay portability) a blinded, randomized, comparative study of *HER2*/CEN-17 ratios measured in 4 formalin-fixed, paraffin-embedded breast cancer specimens was conducted involving 5 different study sites. The 4 specimens included in the study represented varying levels of *HER2* gene amplification and were selected to reflect a natural range of amplification rates, including a non-amplified (measured ratio 0.9-1.2), an altered but non-amplified (measured ratio 1.4-1.7), a low-level amplified (measured ratio 3.0-4.0), and a high level amplified (measured ratio 5.0-8.0). Each site stained and interpreted the 4 specimens in 3 separate runs (total of 12 slides). A provided control was also included in each run.

When stratifying results as either *HER2* gene amplification positive or negative (cut-off ratio = 2.0), there was complete agreement between the 5 sites, see Table 5.

Table 5. Summary of portability results

Specimen	*HER2* negative	*HER2* positive
Non-amplified	15	0
Altered but non-amplified	15	0
Low-level amplification	0	15
High-level amplification	0	15

A day-to-day variation of 10% was found in the 5 laboratories for the *HER2*/CEN-17 ratio.

A site-to-site variation for the *HER2*/CEN-17 ratio of approximately 10-15% was observed for the non-amplified cases and the cases with ratios close to the cut off. This figure is consistent with findings reported in the literature. A higher variation (25%) was observed for the highly amplified specimen; also this is in concordance with the literature, and this variation is not considered clinically relevant (18). As the scoring of highly amplified cases where signals are clustered cannot be based on counting, but must be based on an estimation of the number of signals, a high variation for such cases can be expected.

Clinical utility

HER2 FISH pharmDx™ Kit was investigated in comparative studies with both the PathVision HER-2 DNA Probe Kit and Dako HercepTest™. Results of *HER2* FISH testing are available for a total of 940 breast cancer specimens.

Comparison with PathVysion HER-2 DNA Probe Kit test results

Three studies have been performed that compare the results of the *HER2* FISH pharmDx™ Kit test to the results of the PathVysion HER-2 DNA Probe Kit test. The studies were performed in geographically separate locations, and there was no overlap in the use of specimens. A total of 328 specimens have been tested.

Table 6. Summary data of FISH method comparison studies

Study designation	Concordance study (Danish specimens, N=190)	Concordance study (Japanese specimens, N=52)	Concordance study (French specimens, N=86) (21)*
Concordance (95% confidence interval)	93.68% (90.22% - 97.14%)	96.15%	
Positive percent agreement (95% confidence interval)	86% (77.34% - 95.08%)	97%	
Negative percent agreement (95% confidence interval)	97% (94.05% - 99.89%)	96%	

* Comparison data were provided in the article, but the HER2 FISH assay was not identified.

There were a total of 12 discrepant test results between *HER2* FISH pharmDx™ Kit test and PathVysion™ HER-2 DNA Probe test for the Danish clinical specimens.

Table 7. Summary of data for the 12 discrepant test results.

HER2 FISH(positive)/PathVysion(negative)				*HER2* FISH(negative)/PathVysion(positive)			
ID#	*HER2* FISH ratio	PathVysion ratio	HercepTest score	ID#	*HER2* FISH ratio	PathVysion ratio	HercepTest score
160	2.10* (1.82-2.51)	1.51 (1.39-1.68)	2+	234	1.68 (1.38-1.83)	2.02* (1.84-2.29)	2+
208	3.61 (2.95-4.73)	1.62 (1.51-1.82)	2+	284	1.44 (1.07-1.83)	2.21* (1.94-2.64)	2+
306	2.20* (1.79-2.24)	1.33 (1.18-1.44)	1+	423	1.7 (1.52-1.95)	2.15 (2.02-2.45)	1+
846	2.58 (2.06-3.50)	1.51 (1.42-1.76)	2+	474	1.44 (1.16-1.83)	2.55 (2.38-3.26)	2+
				735	1.68 (1.40-1.99)	2.03* (1.89-2.19)	2+
				746	1.05 (0.96-1.18)	4.53 (4.27-5.17)	3+
				837	1.52 (1.48-1.79)	2.15 (2.10-2.67)	3+
				881	1.83* (1.15-2.69)	2.68 (2.39-3.14)	2+

* CI of mean log ratios included 2.0. Numbers in parentheses are the 95% CI

In this discrepancy analysis, logged ratios were used. The 95% confidence interval was calculated for the 60 logged ratios from the nuclei that were used to calculate the PathVysion HER-2 DNA Probe ratio. For the 4 instances where *HER2* FISH pharmDx™ Kit was positive and PathVysion HER-2 DNA Probe was negative, no interval included the critical value of 2. Of the 8 instances where *HER2* FISH pharmDx™ Kit was negative and PathVysion HER-2 DNA Probe was positive, the 95% CI of 3 (#234, 284 and 735) included the critical value of 2. Similarly, the 95% confidence interval was calculated for the logged ratios from the nuclei that were used to calculate the *HER2* FISH pharm Dx™ Kit ratio. For the 4 instances where *HER2* FISH pharmDx™ Kit was positive and PathVysion HER-2 DNA Probe was negative, 2 included the critical value of 2 (#160 and 306). Of the eight instances where *HER2* FISH pharmDx™ Kit was negative and PathVysion HER-2 DNA Probe was positive, the 95% CI of 1 (#881) included the critical value of 2.

Comparison with HercepTest™ results

Four studies comparing *HER2* FISH pharmDx™ Kit to HercepTest™ results have been conducted. A total of 940 specimens have been compared, using 3+ staining score result as a positive IHC result in the HercepTest™ assay.

Table 8. Summary of *HER2* FISH pharmDx™ Kit and IHC (HercepTest™) comparison studies

Study designation	Danish clinical specimens (N=682)	Japanese specimens (N=52)	French study (N=86) (21)	Danish pilot study (N=120) (20)
Concordance (95% confidence interval)	93.11% (91.21% - 95.01%)	96.15%	87.21%	93.33%
Positive percent agreement (95% confidence interval)	91% (87.39% - 94.57%)	96%	87%	84%
Negative percent agreement (95% confidence interval)	94% (92.12% - 96.46%)	96%	87%	97%

Distribution data of HercepTest™ and *HER2* FISH test results for the Danish clinical specimens are presented in Table 9.

Table 9. Distribution of HER2 status by HercepTest™ and *HER2* FISH pharmDx™ Kit

HercepTest™ staining score	0	1+	2+	3+	Total
N	221	267	84	248	820
%	27	33	10	30	100%
HER2 FISH status					
Amplified	0	8	17	222	247
Non-amplified	106	245	62	22	435
Total FISH tested samples	106	253	79	244	682

Alternative counting methods

Alternative counting methods, including counting a fixed number of events, and conventional counting signals in a fixed number of cells, were evaluated. The alternative counting method implies that more nuclei are counted in cases near the cut off than in highly amplified cases. Statistical analysis using simulation studies on a subset of data with complete 60 cell evaluation for both the Dako HER2 FISH pharmDx™ Kit and the PathVysion HER-2 DNA Probe Kit showed that the alternative counting methods yielded concordance=0.94 when as few as 20 events (minimum 7 nuclei) were counted, and concordance=0.97 when 30 or 60 events (minimum 7 nuclei) were counted. For the two last methods the real number of nuclei counted was less than or equal to 15 and 20, respectively, in 90% of the cases.

In addition, different methods of calculating the *HER2/CEN-17* ratio were evaluated and found to provide equivalent ratios whether using a subset with as few as 20 nuclei being counted. Equivalent ratios were obtained whether the ratio was calculated from the sum of the *HER2* and *CEN-17* counts or by using logarithmically transformed individual ratios that were subsequently averaged.

Troubleshooting - Breast

Problem	Probable Cause	Suggested Action
1. No signals or weak signals	1a. Kit has been exposed to high temperatures during transport or storage	1a. Check storage conditions. Ensure that dry ice was present when the consignment was received. Ensure that vials 2, 3 and 5 have been stored at maximum 2-8 °C, and that vials 3 and 5 have been stored in the dark.
	1b. Microscope not functioning properly - Inappropriate filter set - Improper lamp - Mercury lamp too old - Dirty and/or cracked collector lenses - Unsuitable immersion oil	1b. Check the microscope and ensure that the used filters are suitable for use with the kit fluorochromes, and that the mercury lamp is correct and has not been used beyond expected lifetime. (see Appendix 3). In case of doubt, please contact your local microscope vendor.
	1c. Faded signals	1c. Avoid long microscopic examination and minimize exposure to strong light sources
	1d. Pre-treatment conditions incorrect	1d. Ensure that the recommended pre-treatment temperature and time are used
	1e. Evaporation of Probe Mix during hybridization	1e. Ensure sufficient humidity in the hybridization chamber
2. No green signals	2a. Stringent wash conditions incorrect	2a. Ensure that the recommended stringent wash temperature and time are used, and that coverslips are removed before performing stringent wash
3. No red signals	3a. Pre-treatment conditions incorrect	3a. Ensure that the recommended pre-treatment temperature and time are used
4. Areas without signal	4a. Probe volume too small	4a. Ensure that the probe volume is large enough to cover the area under the coverslip
	4b. Air bubbles caught during Probe Mix application or mounting	4b. Avoid air bubbles. If observed, gently tap them away using forceps

Problem	Probable Cause	Suggested Action
5. Excessive background staining	5a. Inappropriate tissue fixation	5a Ensure that only formalin-fixed, paraffin-embedded tissue sections are used
	5b. Paraffin incompletely removed	5b. Follow the deparaffinization and rehydration procedures outlined in Section B.2
	5c. Stringent wash temperature too low	5c. Ensure that the stringent wash temperature is 65 (±2) °C
	5d. Prolonged exposure of hybridized section to strong light	5d. Avoid long microscopic examination and minimize exposure to strong light
6. Poor tissue morphology	6a. Incorrect Pepsin treatment	6a. Adhere to recommended Pepsin incubation times. See section B.3, step 2. Ensure that the Pepsin is handled at the correct temperature. See Section B.1
	6b. Incorrect pre-treatment conditions may result in unclear or cloudy appearance	6b. Ensure that the recommended pre-treatment temperature and time are used
	6c. Too long Pepsin treatment or very thin section thickness may cause ghost cells or donut cells to appear	6c. Shorten the Pepsin incubation time. See section B.3, step 2. Ensure that the section thickness is 4-6 µm

NOTE: If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please call our Technical Services for further assistance.

Appendix 1 - Breast

HER2 FISH pharmDx™ Kit, Code K5331

Protocol Checklist

Staining Run Log ID:_____

Date (Day 1) of the run: _____
HER2 FISH pharmDx™ Kit, K5331 Lot: _____
Specimen ID: _____
Equipment ID: _____
Date of dilution/expiration of the 1 x Wash Buffer (Vial 6 diluted 1:20):_____/_____

Tissue fixed in neutral buffered formalin	**Yes** ☐ **No** ☐

DAY 1

Step 1: Pre-Treatment	
Date of dilution/expiration of the Pre-Treatment Solution (Vial 1 diluted 1:20)	/
Measured temperature of Pre-Treatment Solution (95-99 °C) if water bath is used for heating	°C
Pre-treatment (10 minutes), and cooling (15 minutes)	
Wash in Wash Buffer (Vial 6 diluted 1:20) (2 x 3 minutes)	
Step 2: Pepsin	
Duration of Pepsin (Vial 2) treatment at 37 °C or	Minutes
Duration of Pepsin (Vial 2) treatment at room temperature	Minutes
Wash in Wash Buffer (Vial 6 diluted 1:20) (2 x 3 minutes)	
Dehydrate slides (3 x 2 minutes) in graded series of ethanol and air dry	
Step 3: *HER2*/CEN-17 Probe Mix	
Apply Probe Mix (Vial 3), coverslip and seal with Coverslip Sealant	
Measured denaturation temperature (82 ±2 °C)	°C
Denaturation for 5 minutes	
Measured hybridization temperature (45 ±2 °C)	°C
Hybridization overnight (protect from light)	

DAY 2

Step 4: Stringent Wash	
Date of dilution/expiration of the Stringent Wash Buffer (Vial 4 diluted 1:20)	/
Measured temperature of Stringent Wash Buffer (65 ±2 °C)	°C
Stringent wash (10 minutes) after removing the coverslips	
Wash in Wash Buffer (Vial 6 diluted 1:20) (2 x 3 minutes)	
Dehydrate slides (3 x 2 minutes) in graded series of ethanol and air dry	
Step 5: Mounting	
Apply 15 µL of Fluorescence Mounting Medium (Vial 5) and coverslip	

Comments:_____

Date and signature, Technician:_____

Appendix 2 - Breast

HER2 FISH pharmDx™ Kit, Code K5331

Scoring Scheme

Staining Run Log ID: _____

Date (Day 1) of the run: _____
HER2 FISH pharmDx™ Kit, K5331 Lot: _____ Specimen ID: _____

Count signals in 20 nuclei					
Nucleus No.	*HER2* score (red)	CEN-17 score (green)	Nucleus No.	*HER2* score (red)	CEN-17 score (green)
1			11		
2			12		
3			13		
4			14		
5			15		
6			16		
7			17		
8			18		
9			19		
10			20		
Total (1-10)			Total (11-20)		

For determination of the *HER2*/CEN-17 ratio, count the number of *HER2* signals and the number of CEN-17 signals in the same 20 nuclei and divide the total number of *HER2* signals by the total number of CEN-17 signals. If the *HER2*/CEN-17 ratio is borderline (1.8-2.2), count an additional 20 nuclei and recalculate the ratio.

A ratio at or near the cut-off (1.8-2.2) should be interpreted with caution (see counting guide).

	HER2	CEN-17	*HER2/CEN-17 ratio*
Total score (1-20)			

❑ Ratio < 2: *HER2* gene amplification was not observed

❑ Ratio > = 2: *HER2* gene amplification was observed

Date and signature, Technician: _____

Date and signature, Pathologist: _____

For scoring guidelines: see Interpretation of Staining.

Appendix 3 - Breast

***HER2* FISH pharmDx™ Kit, Code K5331**

Fluorescence Microscope Specifications

Dako recommends the following equipment for use with the *HER2* FISH pharmDx™ Kit, K5331:

1. **Microscope type**
   - Epifluorescence microscope.
2. **Lamp**
   - 100 watt mercury lamp (keep record of burning time).
3. **Objectives**
   - For screening of the tissue, fluorescence dry 10X or fluorescence oil immersion 16X objectives are applicable.
   - For high power magnification and scoring of signals, only fluorescence oil immersion objectives, e.g. 100X are recommended.
4. **Filters**

Filters are individually designed for specific fluorochromes and must be chosen accordingly. Dako recommends the use of a specific DAPI filter in combination with a high quality Texas Red/FITC double filter.

- DAPI filter, e.g. Chroma filter # 31000.
- Texas Red/FITC double filter, e.g. Omega Optical filter # XF53 or Chroma filter # 51006.
- Texas Red and FITC single filters can be used for confirmation.

Fluorochrome	Excitation Wavelength	Emission Wavelength
FITC	495 nm	520 nm
Texas Red	596 nm	615 nm

Filters are specific to each microscope type and the use of appropriate filters is crucial for the interpretation. If you want detailed information, please contact your microscope provider or your Dako representative.

5. **Oil**
   - Non-fluorescing oil.

Precautions

- A 50 watt mercury lamp is not recommended.
- Rhodamine filters cannot be used.
- Triple filters are not recommended.

A non-optimized microscope may cause problems when reading the fluorescent signals. It is important that the light source has not expired and that it is properly aligned and focused.

Customers should monitor and follow the manufacturer's recommendations for the mercury lamp. The microscope should be maintained and the mercury lamp should be in alignment prior to interpreting results.

An effort should be made to expose the sample to as little of the excitation light as possible in order to minimize fading of the fluorescence.

We recommend that you discuss the set-up of your particular microscope with the manufacturer before starting the fluorescence in situ hybridization, or refer to the literature.

Summary and Explanation - Gastric

The human *HER2* gene (also known as *ERBB2* or *NEU*) is located on chromosome 17 and encodes the HER2 protein or $p185^{HER2}$. The HER2 protein is a membrane receptor tyrosine kinase with homology to the epidermal growth factor receptor (EGFR or HER1) (1-2). The *HER2* gene is present in 2 copies in all normal diploid cells.

Overexpression of the HER2 protein and amplification of the *HER2* gene in gastric cancer have been shown in a large number of studies (reviewed in (21)). HER2 positivity can be detected in approximately 20 % of the patients by either IHC or FISH (21). Preclinical in vitro and in vivo studies have demonstrated that trastuzumab (Herceptin®) is effective in different gastric cancer models, thus leading to the initiation of several clinical studies (21-25).

All of the patients in the phase III BO18255 (ToGA "An open-label, randomized, multicenter, phase III study of traustuzumab in combination with a fluoropyrimidine and cisplatin versus chemotherapy alone as first-line therapy in patients with HER2 positive advanced gastric cancer") study sponsored by Hoffmann-La Roche AG were selected using Dako HercepTest™ (IHC) and Dako *HER2* FISH pharmDx™ Kit (FISH) with HER2 positivity defined as IHC 3+ or FISH+ (*HER2*/CEN-17 ≥ 2.0). The study demonstrated the clinical utility of both HercepTest™ (IHC) and *HER2* FISH pharmDx™ Kit (FISH) for the assessment of HER2 status in patients with advanced gastric or gastroesophageal junction adenocarcinoma for whom trastuzumab treatment is being considered (26).

No patients were enrolled whose tumors were not gene amplified but weakly to strongly HER2 protein overexpressing [FISH(-)/IHC 2+]. It is therefore unclear if patients whose tumors are not gene amplified but HER2 protein-overexpressing [i.e., FISH(-), IHC 2+ or 3+] would benefit from Herceptin® treatment. The study also demonstrated that gene amplification (FISH) and protein overexpression (IHC) are not as correlated as with breast cancer, therefore a single method should not be used to determine HER2 status.

Principle of Procedure - Gastric

HER2 FISH pharmDx™ Kit contains all reagents required to complete a FISH procedure for formalin-fixed, paraffin-embedded tissue section specimens.

After deparaffinization and rehydration, specimens are heated in Pre-Treatment Solution for 10 minutes. The next step involves a proteolytic digestion using ready-to-use Pepsin at room temperature for 5-15 minutes or at 37 °C for 3 minutes. Following the heating and proteolytic pre-treatment steps, this kit employs a ready-to-use FISH Probe Mix based on a combination of PNA (peptide nucleic acid) (12) and DNA technology. This Probe Mix consists of a mixture of Texas Red-labelled DNA probes covering a 218 kb region including the *HER2* gene on chromosome 17; and a mixture of fluorescein-labelled PNA probes targeted at the centromeric region of chromosome 17 (CEN-17). The specific hybridization to the two targets results in formation of a distinct red fluorescent signal at each *HER2* gene locus and a distinct green fluorescent signal at each chromosome 17 centromere. To diminish background staining, the Probe Mix also contains unlabelled PNA blocking probes. After a stringent wash, the specimens are mounted with Fluorescence Mounting Medium containing DAPI and coverslipped. Using a fluorescence microscope equipped with appropriate filters (see Appendix 3), tumor cells are located, and enumeration of the red *(HER2)* and green (CEN-17) signals is conducted. Then the *HER2*/CEN-17 ratio is calculated. Normal cells in the analyzed tissue section will serve as an internal positive control of pre-treatment and hybridization efficiency. For details see the Interpretation of Staining section.

For interactive e-learning please use the *HER2* FISH pharmDx™ e-learning program designed to supply laboratory technicians, pathologists and scientists with an accurate and fast knowledge of how to achieve optimal results using *HER2* FISH pharmDx™ Kit: www.dako.com.

Reagents - Gastric

Materials provided

The materials listed below are sufficient for 20 tests (a test is defined as one 22 mm x 22 mm target area). The number of tests is based on the use of 5-8 drops (250 µL per slide of Vial 2, 10 µL per slide of Vial 3, and 15 µL per slide of Vial 5). The kit provides materials sufficient for 10 individual staining runs.

HER2 FISH pharmDx™ Kit is shipped on dry ice. **To ensure that kit components have not been exposed to high temperatures during transport, dry ice should still be present upon receipt**. Note that some kit components may remain unfrozen, this will not affect the performance of the *HER2* FISH pharmDx™ Kit.

Vial 1	PRE-TREATMENT SOLUTION (20x) **Pre-Treatment Solution (20x)** 150 mL, concentrated 20x MES (2-[*N*-morpholino]ethanesulphonic acid) buffer.
Vial 2	PEPSIN **Pepsin** 7.5 mL, ready-to-use Pepsin solution, pH 2.0; contains stabilizer and an antimicrobial agent.
Vial 3	*HER2*/CEN-17 PROBE MIX ***HER2*/CEN-17 Probe Mix** 0.2 mL, ready-to-use Mix of Texas Red-labelled *HER2* DNA probes and fluorescein-labelled CEN-17 PNA probes; supplied in hybridization buffer with 45% formamide, stabilizer, and unlabelled PNA blocking probes.
Vial 4	STRINGENT WASH BUFFER (20x) **Stringent Wash Buffer (20x)** 150 mL, concentrated 20x SSC (saline-sodium citrate) buffer with detergent.
Vial 5	FLUORESCENCE MOUNTING MEDIUM **Fluorescence Mounting Medium** 0.3 mL, ready-to-use Fluorescence mounting medium with 100 µg/L DAPI (4',6-diamidine-2-phenylindole).
Vial 6	WASH BUFFER (20x) **Wash Buffer (20x)** 500 mL, concentrated 20x Tris/HCl buffer. COVERSLIP SEALANT **Coverslip Sealant** 1 tube, ready-to-use Solution for removable sealing of coverslips.

NOTE: All reagents, including Pre-Treatment Solution, Stringent Wash Buffer, and Fluorescence Mounting Medium, are formulated specifically for use with this kit.

Materials required but not provided

Laboratory reagents

Distilled or deionized water

Ethanol, 96%

Xylene or xylene substitutes

Laboratory equipment

Absorbent wipes

Adjustable pipettes

Calibrated partial immersion thermometer (range 37-100 □C)

Calibrated surface thermometer (range 37-100 □C)

Coverslips (22 mm x 22 mm)

Forceps

Fume hood

Dako Hybridizer (Code S2450)*

Heating block or hybridization oven for denaturation (82 (±2) °C)*

Humid hybridization chamber*

Slides, Dako Silanized Slides, Code S3003, or poly-L-lysine-coated slides (see Specimen Preparation)

Staining jars or baths

Timer (capable of 2-15 minute intervals)

Water bath with lid (capable of maintaining 65 (±2) °C to 99 °C)

Microwave oven with sensing capability if pre-treatment is performed using microwave oven (see B3. Staining protocol. Step 1: Pre-treatment, Method B)

* Heating block or hybridization oven for denaturation (82 (±2) °C) and hybridization (45 (±2) °C) together with a humid hybridization chamber can be used as an alternative to Dako Hybridizer.

Microscope equipment and accessories

Filters for fluorescence microscope: DAPI and FITC/Texas Red double filter, or FITC and Texas Red mono filters - see Appendix 3 for details

Fluorescence microscope with a 100 watt mercury lamp is recommended

Microscope slide folder (cardboard tray for 20 slides with hinged cover or similar)

Precautions - Gastric

1. For in vitro diagnostic use.
2. For professional users.
3. Vial 1, Pre-Treatment Solution (20x), contains 1-<20% 2-morpholinoethanesulphonic acid; Vial 2, Pepsin, contains 5-10% propan-2-ol; Vial 4, Stringent Wash Buffer (20x), contains 1-<5% octoxinol; and Vial 6, Wash Buffer (20x), contains 1-<20% trometamol. At product concentrations these substances do not require hazard labelling. Material Safety Data Sheets (MSDSs) are available for professional users on request.
4. Vial 2, Pepsin, contains pepsin A that may cause an allergic reaction.
5. Vial 3, *HER2*/CEN-17 Probe Mix contains 45% formamide and is labelled:
   Toxic.
   R61 May cause harm to the unborn child.
   S45 In case of accident or if you feel unwell, seek medical advice immediately (show label where possible).
   S53 Avoid exposure – obtain special instructions before use.
   S60 This material and/or its container must be disposed of as hazardous waste.
   As a general rule, persons under 18 years of age are not allowed to work with this product. Users must be carefully instructed in the proper working procedure, the dangerous properties of the product and the necessary safety instructions. Please refer to the Material Safety Data Sheet (MSDS) for additional information.

6. Coverslip Sealant contains 60-100% naphtha (petroleum), hydrotreated light, and is labelled:
   Extremely flammable.
   Dangerous for the environment.
   R11 Highly flammable.
   R51/53 Toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment.
   S9 Keep container in a well-ventilated place.
   S16 Keep away from sources of ignition – No smoking.
   S35 This material and its container must be disposed of in a safe way.
   S57 Use appropriate container to avoid environmental contamination.
   S61 Avoid release to the environment. Refer to special instructions/safety data sheets.
   Please refer to the Material Safety Data Sheet (MSDS) for additional information.
7. Specimens, before and after fixation, and all materials exposed to them, should be handled as if capable of transmitting infection and should be disposed of with proper precautions (16). Never pipette reagents by mouth and avoid contacting the skin and mucous membranes with reagents and specimens. If reagents come in contact with sensitive areas, wash with copious amounts of water.
8. Minimize microbial contamination of reagents to avoid erroneous results.
9. Incubation times and temperatures, or methods other than those specified, may give erroneous results.
10. Tissue fixation methods and thickness of specimen other than those specified may affect tissue morphology and/or signal intensity.
11. Avoid evaporation of *HER2*/CEN-17 Probe Mix during hybridization by ensuring sufficient humidity in the hybridization chamber.
12. Reagents have been optimally diluted. Further dilution may result in loss of performance.
13. Wear appropriate personal protective equipment to avoid contact with eyes and skin. Please refer to the Material Safety Data Sheet (MSDS) for additional information.
14. Due to the heterogeneous nature of gastric cancer specimens it is important to perform a thorough inspection of the entire specimen to evaluate signal distribution before selecting the area for signal enumeration.
15. It is not recommended to evaluate very small specimens, i.e. specimens must have intact morphology and sufficient nuclei for enumeration.
16. If *HER2* FISH analysis is performed on a biopsy specimen, multiple (7-8) evaluable biopsies from different regions of the tumor should be analyzed to ensure reliable determination of HER2 status.
17. For identification of all tissue cores in a biopsy sample it is important to inspect the H&E stained slide
18. *HER2* gene amplification and HER2 protein overexpression are not as well correlated in gastric cancer as with breast cancer; therefore a single method should not be used to determine HER2 status.

Storage - Gastric

Store in the dark at 2-8 °C. All reagents tolerate frozen storage. Freezing and thawing the reagents for each analysis does not affect performance.

The Pepsin, *HER2*/CEN-17 Probe Mix, and Fluorescence Mounting Medium (Vials 2, 3 and 5) may be affected adversely if exposed to heat. Do not leave these components at room temperature. The *HER2*/CEN-17 Probe Mix and Fluorescence Mounting Medium (Vials 3 and 5) may be affected adversely if exposed to excessive light levels. Do not store these components or perform analysis in strong light, such as direct sunlight.

Do not use the kit after the expiration date stamped on the kit box. If reagents are stored under conditions other than those specified in this package insert, the user must validate reagent performance (13).

There are no obvious signs to indicate instability of this product. Therefore, it is important to evaluate normal cells in the analyzed tissue section. If an unexpected fluorescence pattern is observed which cannot be explained by variations in laboratory procedures, and a problem with the *HER2* FISH pharmDx™ Kit is suspected, contact our Technical Services.

Specimen Preparation - Gastric

Gastric or gastroesophageal junction adenocarcinoma specimens from biopsies, excisions or resections must be handled to preserve the tissue for FISH analysis. Standard methods of tissue processing for immunohistochemical staining should be used for all specimens (14). When testing small biopsy specimens, ascertain intact tumor morphology and the presence of sufficient nuclei for enumeration. If *HER2* FISH analysis is performed on a biopsy specimen, multiple (7-8) evaluable biopsies from different regions of the tumor should be analyzed to ensure reliable determination of HER2 status.

Paraffin-embedded sections
Only tissue preserved in neutral buffered formalin and paraffin-embedded are suitable for use. Specimens should e.g. be blocked into a thickness of 3 or 4 mm and fixed for 18-24 hours in neutral buffered formalin. Biopsy specimens were fixed for 6-8 hours in the ToGA trial [for study reference, refer to (26)]. The tissues are then dehydrated in a graded series of ethanol and xylene, followed by infiltration by melted paraffin held at no more than 60 °C. Properly fixed and embedded tissues will keep indefinitely prior to sectioning and slide mounting if stored in a cool place (15-25 °C) (14-15). Other fixatives are not suitable.

Tissue specimens should be cut into sections of 3-6µm.

The slides required for *HER2* gene amplification analysis and verification of tumor presence should be prepared at the same time. A minimum of 2 serial sections is recommended, 1 section for tumor presence stained with hematoxylin and eosin (H&E stain), and 1 section for *HER2* gene amplification analysis. It is recommended that tissue sections are mounted on Dako Silanized Slides, Code S3003, or poly-L-lysine-coated slides. Specimens should be analyzed within 4-6 months of sectioning when stored at room temperature (20-25 °C).

INSTRUCTIONS FOR USE - Gastric

A. Reagent Preparation - Gastric
It is convenient to prepare the following reagents prior to staining:

A.1 Pre-Treatment Solution
Crystals may occur in Vial 1, but they will dissolve at room temperature. Ensure that no crystals are present before preparation of reagent.

Dilute a sufficient quantity of Vial 1 (Pre-Treatment Solution 20x) by diluting the concentrate 1:20 in distilled or deionized water. Unused diluted solution may be stored at 2-8 °C for one month. Discard diluted solution if cloudy in appearance.

A.2 Stringent Wash Buffer
Dilute a sufficient quantity of Vial 4 (Stringent Wash Buffer 20x) by diluting the concentrate 1:20 in distilled or deionized water. Unused diluted buffer may be stored at 2-8 °C for one month. Discard diluted buffer if cloudy in appearance.

A.3 Wash Buffer
Dilute a sufficient quantity of Vial 6 (Wash Buffer 20x) by diluting the concentrate 1:20 in distilled or deionized water. Unused diluted buffer may be stored at 2-8 °C for one month. Discard diluted buffer if cloudy in appearance.

A.4 Ethanol series

From a 96% ethanol solution, prepare 3 jars with 70%, 85%, and 96% ethanol, respectively. Store covered jars at room temperature or at 2-8 °C, and use for a maximum of 200 slides. Discard solutions if cloudy in appearance.

B. Staining Procedure - Gastric

B.1 Procedural notes

The user should read these instructions carefully and become familiar with all components prior to use (see Precautions).

If kit components are stored frozen, it is recommended to move the reagents to 2-8 °C the day before performing the analysis to allow proper temperature equilibration. All reagents should be equilibrated to the relevant temperature prior to use as follows:

Vial 1: The diluted Pre-Treatment Solution should be equilibrated to **95-99 °C** if water bath is used for pre-treatment (B3. Staining protocol, Step 1: Pre-Treatment Method A). If microwave oven with sensing capability is used for pre-treatment (B3. Staining protocol, Step 1: Pre-Treatment, Method B) the diluted Pre-Treatment Solution should be equilibrated to room temperature **20-25 °C**.

Vial 2: Pepsin should be applied **at 2-8 °C** and kept cold continuously.

Vial 3: *HER2*/CEN-17 Probe Mix may be applied at any temperature from **2-25 °C.**

Vial 4: The Diluted Stringent Wash Buffer; one jar should be equilibrated to room temperature, another jar should be equilibrated to **65 (±2) °C** prior to use.

Vial 5: Fluorescence Mounting Medium may be applied at any temperature from **2-25 °C.**

Vial 6: The Diluted Wash Buffer should be equilibrated to room temperate **20-25 °C.**

The Coverslip Sealant may be applied at any temperature from **2-25 °C.**

All steps must be performed at the outlined temperature.

The procedure includes a number of dehydrations followed by drying of the tissue sections. Ensure that tissue sections are completely dry before proceeding to the next step. Do not allow tissue sections to dry during the other procedural steps.

If the staining procedure has to be interrupted, slides may be kept in Wash Buffer after the deparaffinization step for up to 1 hour at room temperature (20-25 °C) without affecting the results.

B.2 Treatment of tissues prior to staining

Deparaffinization and rehydration: Prior to performing the analysis, tissue slides must be deparaffinized to remove embedding medium and rehydrated. Avoid incomplete removal of paraffin. Residual embedding medium will result in increased non-specific staining. This step should be performed at room temperature (20-25 °C).

1. Place slides in a xylene bath and incubate for 5 (±1) minutes. Change baths and repeat once.
2. Tap off excess liquid and place slides in 96% ethanol for 2 (±1) minutes. Change baths and repeat once.
3. Tap off excess liquid and place slides in 70% ethanol for 2 (±1) minutes. Change baths and repeat once.
4. Tap off excess liquid and place slides in diluted Wash Buffer (see INSTRUCTIONS FOR USE, Section A.3) for a minimum of 2 minutes. Commence staining procedure as outlined in Section B.3, Step 1, Pre-Treatment.

Xylene and alcohol solutions should be changed after 200 slides or less.

Xylene substitutes may be used.

NOTE: The reagents and instructions supplied in this kit have been designed for optimal performance. Further dilution of the reagents or alteration of incubation temperatures may give

erroneous or discordant results. Differences in tissue processing and technical procedures in the user's laboratory may invalidate the assay results.

B.3 Staining protocol

DAY 1

Step 1: Pre-Treatment

Pre-treatment can be performed either by using water bath as described in method A) or, alternatively, by use of microwave oven with sensing capability as described in method B)

Method A) Pre-treatment using water bath:

Fill staining jars, e.g. Coplin jars, with the diluted Pre-Treatment Solution (see INSTRUCTIONS FOR USE, Section A.1). Place staining jars containing Pre-Treatment Solution in water bath. Heat water bath and the Pre-Treatment Solution to 95-99 °C. Measure temperature inside jar with a calibrated thermometer to ensure correct temperature. Cover jars with lids in order to stabilize the temperature and avoid evaporation.

Immerse the room temperature deparaffinized sections into the preheated Pre-Treatment Solution in the staining jars. Re-check temperature and incubate for 10 (±1) minutes at 95-99 °C.

Remove the entire jar with slides from the water bath. Remove lid and allow the slides to cool in the Pre-Treatment Solution for 15 minutes at room temperature.

Transfer the slides to a jar with diluted Wash Buffer (see INSTRUCTIONS FOR USE, Section A.3) for 3 minutes at room temperature (20-25 °C).

Replace Wash Buffer and soak sections for another 3 minutes.

Method B) Pre-Treatment using microwave oven with sensing capability:

Fill a plastic jar with diluted room temperature (20-25 °C) Pre-Treatment Solution. Immerse the deparaffinized sections in Pre-Treatment Solution, cover the jar with a punctured lid and place it in the microwave oven. Select the boiling sensor function and a program that runs for 10 minutes after boiling temperature has been reached*.

Following the 10 minutes incubation take the jar with slides out of the oven, remove the lid and cool for 15 minutes at room temperature. Transfer the slides to a jar with diluted Wash Buffer and soak for 3 minutes at room temperature (20-25 °C). Replace Wash Buffer and soak sections for another 3 minutes.

*The use of a microwave oven with a sensing capability means that the oven must include a sensor and programs which initially heat the Pre-Treatment Solution to the boiling point and subsequently maintain the required pre-treatment temperature (above 95 °C) while counting down the preset time (10 (±1) minutes). Some microwave oven models with sensing capability may not include the possibility to freely set a count-down time. If the model only includes pre-set programs, be sure to select a program which maintain the required pre-treatment temperature (above 95 °C) for at least 10 (±1) minutes and manually stop the program after 10 (±1) minutes.

NOTE: The Pre-Treatment Solution is designed for a single use application only. Do not re-use.

Step 2: Pepsin, ready-to-use

Pepsin incubation can be performed either at room temperature (20-25 °C) as described in method A) or, alternatively, at 37 °C as described in method B)

Tap off excess buffer. Using lintless tissue (such as an absorbent wipe or gauze pad), carefully wipe around the specimen to remove any remaining liquid and to keep reagents within the prescribed area.

Apply 5-8 drops (250 µL) of cold (2-8 °C) Pepsin (Vial 2) to cover specimen. Always store Pepsin at 2-8 °C.

Method A) Incubation at room temperature:
Incubate for 5-15 minutes at room temperature (20-25 °C). An incubation time of 10 minutes will be adequate for most specimens, but the optimal incubation time may depend on tissue fixation and/or thickness of specimen and should be determined by the user.

Tap off Pepsin and soak sections in the diluted Wash Buffer (see INSTRUCTIONS FOR USE, Section A.3) for 3 minutes at room temperature (20-25 °C).

Replace diluted Wash Buffer and soak sections for another 3 minutes. Continue to dehydration.

Method B) Incubation at 37 °C:
Place specimen with Pepsin on a heating block at 37 °C – e.g. Dako Hybridizer – and incubate for 3 minutes. An incubation time of 3 minutes will be adequate for most specimens, but the optimal incubation time may depend on tissue fixation and/or thickness of specimen and should be determined by the user.

Tap off Pepsin and soak sections in diluted Wash Buffer for 3 minutes at room temperature (20-25 °C).

Replace Wash Buffer and soak sections for another 3 minutes. Continue to dehydration.

Dehydrate tissue sections through a graded series of ethanol: 2 minutes in 70% ethanol, 2 minutes in 85% ethanol, and 2 minutes in 96% ethanol.

Allow tissue sections to air dry completely.

Step 3: *HER2*/CEN-17 Probe Mix, ready-to-use
The following step should be performed in a fume hood.

Apply 10 µL of *HER2*/CEN-17 Probe Mix (Vial 3) to the centre of the tissue section. Immediately place a 22 mm x 22 mm glass coverslip over the Probe Mix and allow it to spread evenly under the coverslip. Avoid air bubbles. If air bubbles are observed, gently tap them away from the tissue using forceps.

Seal coverslip with Coverslip Sealant by ejecting the Sealant around the periphery of the coverslip. Allow the Coverslip Sealant to overlap the coverslip and the slide, thereby forming a seal around the coverslip. Make sure that the Coverslip Sealant covers the entire edge of the coverslip.

Prepare Dako Hybridizer* (Code S2450) for a hybridization run. Make sure that Humidity Control Strips (Code S2452) are saturated and optimal for use. Start the Hybridizer and choose a program that will denature at 82 °C for 5 minutes and hybridize overnight (14-20 hours) at 45 °C (please refer to Dako Hybridizer Instruction Manual for details). Place slides in the Hybridizer, make sure the lid is properly closed and start program.

*Instrumentation that allows for conditions similar to the ones described above may be used for denaturation and hybridization:
Place slides on a flat metal or stone surface (heating block or on a block in a hybridization oven) preheated to 82 (±2) °C. Denature for 5 minutes ensuring that the temperature of the block does not drop below 80 °C at any time.

Place slides in a preheated humidified hybridization chamber. Cover the chamber with a lid and incubate overnight (14-20 hours) at **45 (±2) °C**. Please note that a hybridization temperature of 37 °C is not suitable for use with the probes contained within this kit.

DAY 2

Step 4: Stringent Wash
Fill two staining jars, e.g. Coplin jars, with the diluted Stringent Wash Buffer (see INSTRUCTIONS FOR USE, Section A.2). A minimum volume of 100 mL or 15 mL per slide in each jar is recommended.

Place one of the staining jars containing diluted Stringent Wash Buffer at room temperature in a fume hood and the other in a water bath. Heat water bath and the diluted Stringent Wash Buffer to 65 (±2) °C. Ensure that the temperature has stabilized. Cover jar with lid in order to stabilize the temperature and avoid evaporation. Measure temperature inside the water bath jar with a calibrated thermometer to ensure correct temperature. The Stringent Wash Buffer contains detergent and may become turbid at 65 °C; this will not affect performance.

Using forceps or gloves, take slides from the hybridization chamber and gently remove Coverslip Sealant as well as coverslip and place slides in the room temperature pre-wash jar, one at a time.

As soon as all coverslips have been removed, transfer slides from the room temperature, pre-wash jar to the 65 (±2) °C jar in the water bath. Perform stringent wash for exactly 10 minutes at 65 (±2) °C.

Remove slides from the diluted Stringent Wash Buffer, and soak sections in diluted Wash Buffer for 3 minutes at room temperature (20-25 °C).

Change diluted Wash Buffer and soak sections for another 3 minutes.

Dehydrate tissue sections through a graded series of ethanol: 2 minutes in 70% ethanol, 2 minutes in 85% ethanol, and 2 minutes in 96% ethanol.

Allow tissue sections to dry completely.

Step 5: Mounting
Apply 15 µL of Fluorescence Mounting Medium containing DAPI (Vial 5) to the target area of the slide and apply a glass coverslip.

NOTE: Slides may be read after 15 minutes or within 7 days after mounting. However, fading occurs if slides are exposed to light or high temperatures. To minimize fading, store slides in the dark at 2-8 °C.

Quality Control - Gastric

1. Signals must be bright, distinct and easy to evaluate.
2. Normal cells allow for an internal control of the staining run.
   - Normal cells should have 1-2 clearly visible green signals indicating that the CEN-17 PNA Probe has successfully hybridized to the centromeric region of chromosome 17.
   - Normal cells should also have 1-2 clearly visible red signals indicating that the *HER2* DNA Probe has successfully hybridized to the *HER2* amplicon.
   - Due to tissue sectioning, some normal cells will have less than the expected 2 signals of each colour.
   - Failure to detect signals in normal cells indicates assay failure, and results should be considered invalid.
3. Nuclear morphology must be intact when evaluated using a DAPI filter. Numerous ghost like cells and a general poor nuclear morphology indicate over-digestion of the specimen, resulting in loss or fragmentation of signals. Such specimens should be considered invalid.
4. The minimum number of assessable tumor cells is 20.
5. Differences in tissue fixation, processing, and embedding in the user's laboratory may produce variability in results, necessitating regular evaluation of in-house controls.

Interpretation of Staining - Gastric

Assessable tissue
Locate the tumor within the context of the H&E stained slide and evaluate the same area on the FISH stained slide (in the DAPI filter). Only specimens from patients with gastric or gastroesophageal junction adenocarcinoma should be analyzed. In cases with intestinal metaplasia and adenocarcinoma in the same specimen, only the carcinoma component should be scored. Avoid areas of heavy inflammation, necrosis and areas where the nuclear borders are ambiguous. Do not include nuclei that require subjective judgment. Do not include nuclei with weak signal intensity and non-specific or high background.

Begin with a microscope evaluation of the complete FISH stained section and the area assigned on the H&E section, respectively. Before enumeration of the FISH stained section, note the overall signal distribution (homogenous or heterogeneous) on the signal enumeration sheet. In

case of heterogeneous distribution, note whether focal amplification or single cell amplification (mosaic) is present.

1) Homogenous signal distribution
In case the signal distribution is homogenous, enumerate the number of chromosome centromeres (green signals) and the number of *HER2* genes (red signals) respectively, from 20 cells in 1-2 representative tumor areas.

2) Heterogeneous signal distribution
In case the signal distribution is heterogeneous, enumerate a total of 20 cells from selected areas as specified below:
A) If focal amplification exists, areas with amplified cells should be selected
B) If mosaic distribution or amplified, polysomal and disomal cells are present, count in areas with amplified cells. Within these areas, not only amplified cells but also adjacent non-amplified cells should be counted for a total of 20 cells.

If possible, do not select overlapping areas.

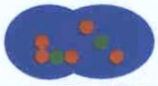
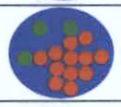
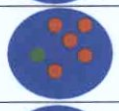
Disregard staining of bacterial DNA
A number of specialized cells (mast cells and macrophages), present interspersed in the gastric tissue, exhibit a high level of staining by the *HER2* probe due to presence of bacterial DNA. This results in highly red fluorescent cells that are clearly distinct from tumor cells with high *HER2* gene amplification.

Signal enumeration: When an area has been selected for signal evaluation, begin analysis in one of the 20 adjacent chosen nuclei and then count in a cell-by-cell fashion only leaving out nuclei that do not meet the quality criteria. Locate the tumor within the context of the H&E stained slide and evaluate the same area on the FISH stained slide. Scan the entire stained slide to account for possible heterogeneity. Select an area having good nuclei distribution. Begin analysis in the upper left quadrant of the selected area and, scanning from left to right, count the number of signals within the nuclear boundary of each evaluated nucleus according to the guidelines below (see also Appendix 7).

- Focus up and down to find all of the signals in the individual nucleus.
- Count two signals that are the same size and separated by a distance equal to or less than the diameter of the signal as only one signal. The distance has to be at least equal to the diameter of one normal-sized signal in order to count two individual signals. When the distance between two signals is less than the diameter of a signal it is counted as one.
- In nuclei with high levels of *HER2* gene amplification, the *HER2* signals may be positioned very close to each other forming a cluster of signals. In these cases the number of *HER2* signals cannot be counted, but must be estimated. Special attention must be paid to the green signals, as clusters of red *HER2* signals can cover the green signals making them impossible to see. In case of doubt, please check the green signals using a specific FITC filter.

Do not score nuclei without signals or with signals of only one colour. Score only those nuclei with one or more FISH signals of each colour.

Signal counting guide

1		Do not count. Nuclei are overlapping, not all areas of nuclei are visible
2		Two green signals, do not score nuclei with signals of only one color
3		Count as 3 green and 12 red signals (cluster estimation)
4		Count as 1 green and 1 red signal. Two signals of the same size and separated by a distance equal to or less than the diameter of one signal are counted as one
5		Do not count (over- or underdigested nuclei). Missing signals in the centre of nuclei (donut-shaped nuclei).
6		Count as 2 green and 3 red signals. Two signals of the same size and separated by a distance equal to or less than the diameter of one signal are counted as one
7		Count as 1 green and 5 red signals
8		Count as 3 green (1 green out of focus) and 3 red signals
9		Cluster of red signals hiding green signals, check the green signals with a specific FITC filter, or do not count

Record counts in a table as shown in Appendix 5-6.

Count 20 nuclei per tissue specimen, when possible from distinct tumor areas.

Calculate the *HER2*/CEN-17 ratio by dividing the total number of red *HER2* signals by the total number of green CEN-17 signals.

Specimens with a *HER2*/CEN-17 ratio above or equal to 2 should be considered *HER2* gene amplified (27).

Results at or near the cut-off (1.8-2.2) should be interpreted with caution.

If the ratio is borderline (1.8-2.2), count an additional 40 nuclei and calculate the ratio for the 40 nuclei. If the enumeration continues to be borderline, the result of the second evaluation is valid. If available, the immunohistochemical staining of HER2 should be included for better orientation during the second enumeration.

In case of doubt, the specimen slide should be re-scored. For borderline cases a consultation between the pathologist and the treating physician is warranted.

Limitations - Gastric

1. FISH is a multi-step process that requires specialized training in the selection of the appropriate reagents, as well as in tissue selection, fixation, and processing, preparation of the FISH slide, and interpretation of the staining results.
2. FISH results are dependent on the handling and processing of the tissue prior to staining. Improper fixation, washing, drying, heating, sectioning, or contamination with other tissues or fluids may influence on probe hybridization. Inconsistent results may be due to variations in fixation and embedding methods, or to inherent irregularities within the tissue.
3. For optimal and reproducible results, the tissue slides must be deparaffinized completely. The paraffin removal needs to be completed at the beginning of the staining process. (See INSTRUCTIONS FOR USE, section B.2).
4. Only temperature-calibrated water bath, heating block, and hybridization oven should be used. Use of other types of equipment may result in evaporation of *HER2*/CEN-17 Probe Mix during hybridization and must be validated by user.

Performance Characteristics - Gastric

Background and clinical data

The clinical safety and efficacy of trastuzumab (Herceptin®) has been demonstrated in the BO18255 study (the ToGA trial "An open-label, randomized, multicenter, phase III study of traustuzumab in combination with cisplatin and a fluoropyrimidine (capecitabine or 5-Fluorouracil) (FC+H) versus chemotherapy (FC) alone as first-line therapy in patients with HER2 positive advanced gastric cancer") (26). The study was designed as an open labeled, randomized, multicenter phase III study in HER2-positive patients with advanced gastric or gastroesophageal junction adenocarcinoma. In the BO18255 study HER2 positivity was defined as being either IHC-positive (3+) using HercepTest™ (Dako) and/or FISH positive (*HER2*/CEN-17 ≥ 2.0) using *HER2* FISH pharmDx™ Kit (Dako). After inclusion in the study the patients were randomized to receive chemotherapy (5-FU or capecitabine and cisplatin) or chemotherapy plus trastuzumab.

The main efficacy outcome measure was overall survival (OS) analyzed by stratified log rank test. The final OS analysis based on 351 deaths was statistically significant (nominal significance level of 0.0193). An updated OS analysis was also conducted at one year after the final analysis. The median overall survival of 13.5 months on the Herceptin® plus chemotherapy arm was significantly longer compared to 11.0 month median overall survival on the chemotherapy alone arm. The efficacy results of both the final and the updated analyses are summarized in Table 10 and Figure 1.

Table 10. Overall Survival in Intention to Treat (ITT) Population

	FC Arm N= 296	FC + H Arm N=298
Final Overall Survival		
No. Deaths (%)	184 (62.2%)	167 (56.0%)
Median	11.0	13.5
95% CI (mos.)	(9.4, 12.5)	(11.7, 15.7)
Hazard Ratio	0.73	
95% CI	(0.60, 0.91)	
p-value*, two-sided	0.0038*	
Updated Overall Survival		
No. Deaths (%)	227 (76.7%)	221 (74.2%)
Median	11.7	13.1
95% CI (mos.)	(10.3, 13.0)	(11.9, 15.1)
Hazard Ratio	0.80	
95% CI	(0.67, 0.97)	

*Comparing with the nominal significance level of 0.0193

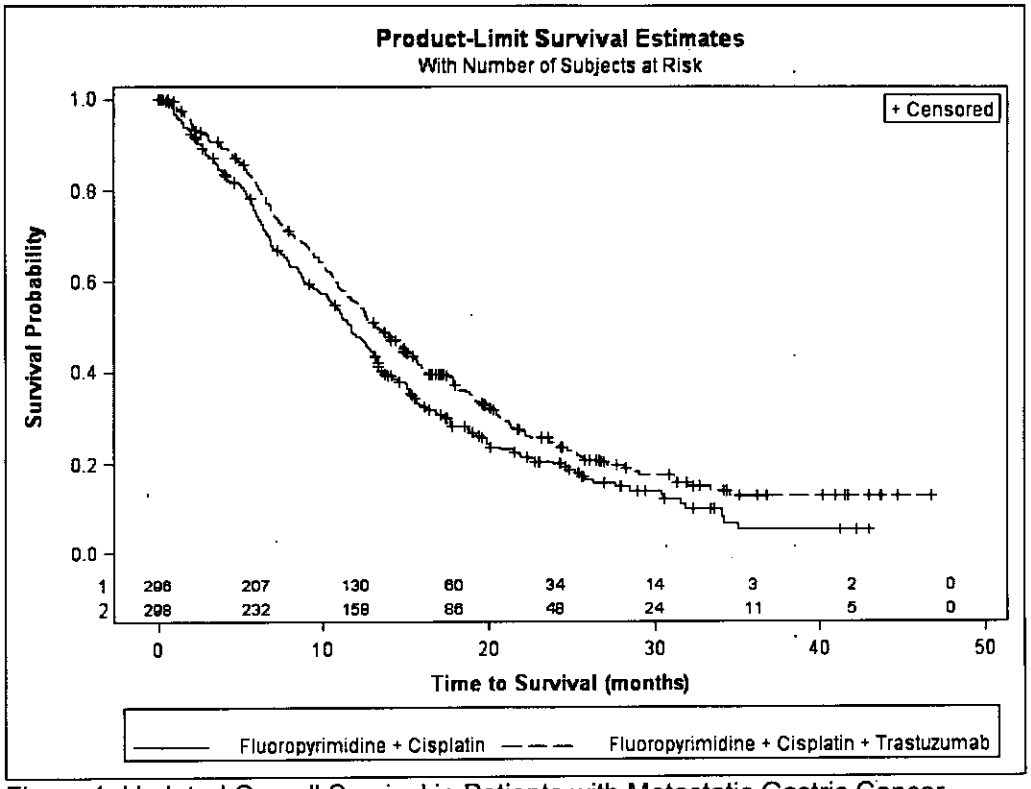


Figure 1. Updated Overall Survival in Patients with Metastatic Gastric Cancer

An exploratory analysis of OS based on gene amplification (FISH) and protein-overexpression (IHC) testing is summarized in Table 11.

Table 11. Exploratory Analyses by HER2 Status with Updated Overall Survival Results.

	FC N=296[a]	FC+H N=298[b]
FISH+ / IHC 0, 1+ subgroup (N=133)		
No. Deaths / n (%)	57/71 (80.3%)	56/62 (90.3%)
Median OS Duration (mos.)	8.8	8.3
95% CI (mos.)	(6.4, 11.7)	(6.2, 10.7)
Hazard ratio (95% CI)	1.33 (092, 1.92)	
FISH+ / IHC2+ subgroup (N=160)		
No. Deaths / n (%)	65/80 (81%)	64/80 (80%)
Median OS Duration (mos.)	10.8	12.3
95% CI (mos.)	(6.8, 12.8)	(9.5, 15.7)
Hazard ratio (95% CI)	0.78 (0.55, 1.10)	
FISH+ or FISH-/IHC3+[c] subgroup (N=294)		
No. Deaths / n (%)	104/143 (73%)	96/151 (64%)
Median OS Duration (mos.)	13.2	18.0
95% CI (mos.)	(11.5. 15.2)	(15.5, 21.2)
Hazard ratio (95% CI)	0.66 (0.5, 0.87)	

Median survival was estimated from Kaplan-Meier curves.

[a] Two patients on FC arm who were FISH+ but IHC status unknown were excluded from the analyses.

[b] Five patients on Herceptin® arm who were FISH+ but IHC status unknown were excluded from the analyses.

[c] Includes 6 patients on chemotherapy arm, 10 patients on Herceptin® arm with FISH-, IHC3+ and 8 patients on chemotherapy arm, 8 patients on Herceptin® arm with FISH status unknown, IHC3+.

Hybridization efficiency

Hybridization efficiency of *HER2* FISH pharmDx™ Kit was investigated as part of the reproducibility study. From the total 360 formalin-fixed, paraffin-embedded tissue sections tested at the three study sites 358 could be enumerated in accordance with product guidelines. Thus, the hybridization efficiency was 99.4%.

Analytical sensitivity

The analytical sensitivity of the *HER2*/CEN-17 Probe Mix when used on gastric cancer tissue was investigated using 18 gastric cancer adenocarcinoma specimens. The ratio between the number of *HER2* signals and CEN-17 signals was calculated based on a counting of 20 nuclei from normal cells surrounding the tumor. The *HER2*/CEN-17 ratio was scored between 0.91 and 1.09 which was within the pre-set acceptance criteria.

Analytical specificity

The *HER2* DNA probes in the *HER2*/CEN-17 Probe Mix have been end-sequenced and mapped to confirm a total coverage of 218 kb including the *HER2* gene.

The CEN-17 PNA probes in the *HER2*/CEN-17 Probe Mix have been tested individually and in combination to confirm their specific hybridization to the centromeric region of chromosome 17.

To exclude cross-hybridization to chromosomes other than chromosome 17, studies were performed on metaphase spreads according to standard Dako QC procedures. A total of 250 metaphase spreads were evaluated for specific hybridization of the *HER2* DNA and CEN-17 PNA probe mixes. In all 250 cases the hybridization was specific for chromosome 17. No cross-hybridization to loci on other chromosomes was observed in any of the 250 cases.

Robustness studies
The robustness of *HER2* FISH pharmDx™ assay was tested by varying pre-treatment time and temperature, pepsin incubation time, denaturation temperature, hybridization time and temperature, and stringent wash time, temperature and buffer concentration.

No significant difference in results was observed at the following experimental conditions:

- Pre-treatment for 7, 9, 10, 11, and 13 minutes at 95-97 °C
- Pre-treatment at 89, 92 and 95-97 °C for 10 minutes
- Pepsin incubation times of 2, 2½, 3 and 4 minutes at 37 °C
- Denaturation temperatures of 72, 77, 82, 87 and 92 °C for 5 minutes
- Hybridization times 10, 12 and 14 hours
- Hybridization temperatures at 40, 45 and 50 °C
- Stringency wash for 5, 10 and 15 minutes at 65 °C
- Stringency wash at 60, 65 and 70 °C for 10 minutes
- Stringency wash buffer concentration at 1:10, 1:15, 1:20, 1:30 and 1:40

NOTE: For the robustness tests only one parameter in the staining procedure was changed at a time while all other parameters were kept constant. It is recommended to adhere to the time and temperatures indicated in the staining procedure.

Repeatability
The repeatability of the *HER2*/CEN-17 ratio was investigated with *HER2* FISH pharmDx™ Kit using three consecutive sections from 9 different gastric cancer adenocarcinoma specimens. The coefficient of variance was found to be 1-5% within the preset acceptance criteria.

Repeatability on consecutive sections of gastric adenocarcinoma specimens (IHC 2+) with different thickness (2-7 µm) was tested with the *HER2* FISH pharmDx™ Kit. The coefficient of variance of the *HER2*/CEN-17 ratio in this study was found to be 2-6% i.e. in the same range as for tissue of equal thickness and within the preset acceptance criteria.

Reproducibility
The day-to-day, site-to-site and observer-to-observer reproducibility was tested using *HER2* FISH pharmDx™ Kit. Sections from 24 different gastric cancer specimens obtained from stomach (75%) or gastroesophageal junction (25%) were stained and analyzed on five non-consecutive days by two observers at three sites. This resulted in a total of 720 signal enumerations of the *HER2*/CEN-17 ratio (see Table 12). The specimens represented surgical resections (70%) and biopsies (30%) with an equal number of non-amplified, IHC 2+ (determined by HercepTest™), and amplified specimens.

Table 12. *HER2*/CEN-17 ratio determinations from 720 signal enumerations

	Site One Observers		**Site Two** Observers		**Site Three** Observers		
	A	B	C	D	E	F	Total
Day 1	24	24	24	24	24	24	**144**
Day 2	24	24	24	24	24	24	**144**
Day 3	24	24	24	24	24	24	**144**
Day 4	24	24	24	24	24	24	**144**
Day 5	24	24	24	24	24	24	**144**
Total	**120**	**120**	**120**	**120**	**120**	**120**	**720**

The average CV (Standard Deviation / Mean x 100) for the *HER2*/CEN-17 ratio determined for each observer from the eight specimens in each category (five observations per block) is seen in Table 13. The overall average CVs for each observer were 5.4%, 3.8%, 12.0%, 11.9%, 4.7% and 24.4%, respectively (Table 13). The average CVs determined in each category for all combined observations (days, sites, observers) were 22.8%, 16.5% and 25.2% in the non-amplified, IHC 2+, and amplified category, respectively.

Table 13. The average day-to-day CV (%) for each observer and the average CV (%) for all enumerations in the three categories.

Average CVs (%)	Site One Observers		Site Two Observers		Site Three Observers		All Sites All Observers
	A	B	C	D	E	F	
Non-	5.4	4.7	8.1	16.3	3.1	20.6	22.8
IHC 2+	6.2	3.9	8.9	7.5	4.5	17.2	16.5
Amplified	4.7	2.9	19.1	11.9	6.6	35.3	25.2
All	**5.4**	**3.8**	**12.0**	**11.9**	**4.7**	**24.4**	**21.5**

Table 13 shows the average CV (%) for each observer at the three sites (day-to-day variation) for determinations of the *HER2*/CEN-17 ratios. In the last column the average CV (%) is given for all determinations made for each specimen in the three categories. Using statistical variance component models it was found that the variation associated with the specimen itself contributed to the vast majority of the day-to-day, site-to-site and observer-to-observer variation.
Table 14 shows the mean *HER2*/CEN-17 ratio and CV (%) for each of the specimens included in the study. For each specimen the mean and CV is derived from the total of 30 enumerations made by the six different observers on five different days.

Table 14. Mean and CV (%) of *HER2*/CEN-17 ratios obtained for each specimen

Specimen	*HER2*/CEN-17 Ratio Non-amplified		Specimen	*HER2*/CEN-17 Ratio IHC 2+		Specimen	*HER2*/CEN-17 Ratio Amplified	
	Mean	CV (%)		Mean	CV (%)		Mean	CV (%)
54210	1.27	15.04	**53116**	3.04	18.44	**59248**	21.07	25.48
54220	1.28	16.89	**53832**	1.13	11.71	**59249**	14.7	20.73
58752	1.39	20.42	**58882**	1.21	14.98	**59257**	9.89	35.33
59252	1.09	8.15	**58892**	1.12	13.97	**59269**	8.73	21.62
59259	1.2	28.99	**59239**	2.21	27.59	**59272**	11.31	23.98
59262	1.23	16.23	**59241**	1.14	10.97	**59300**	6.67	19.85
59297	1.66	65.46	**59246**	2.85	17.76	**59302**	7.8	33.83
59314	1.14	11.41	**59254**	1.32	16.31	**59304**	22.96	20.57

Troubleshooting - Gastric

Problem	Probable Cause	Suggested Action
1. No signals or weak signals	1a. Kit has been exposed to high temperatures during transport or storage	1a. Check storage conditions. Ensure that dry ice was present when the consignment was received. Ensure that vials 2, 3 and 5 have been stored at maximum 2-8 °C, and that vials 3 and 5 have been stored in the dark.
	1b. Microscope not functioning properly - Inappropriate filter set - Improper lamp - Mercury lamp too old - Dirty and/or cracked collector lenses - Unsuitable immersion oil	1b. Check the microscope and ensure that the used filters are suitable for use with the kit fluorochromes, and that the mercury lamp is correct and has not been used beyond expected lifetime. (see Appendix 7). In case of doubt, please contact your local microscope vendor.
	1c. Faded signals	1c. Avoid long microscopic examination and minimize exposure to strong light sources.
	1d. Pre-treatment conditions incorrect	1d. Ensure that the recommended pre-treatment temperature and time are used.
	1e. Evaporation of Probe Mix during hybridization	1e. Ensure sufficient humidity in the hybridization chamber
2. No green signals	2a. Stringent wash conditions incorrect	2a. Ensure that the recommended stringent wash temperature and time are used, and that coverslips are removed before performing stringent wash
3. No red signals	3a. Pre-treatment conditions incorrect	3a. Ensure that the recommended pre-treatment temperature and time are used
4. Areas without signal	4a. Probe volume too small	4a. Ensure that the probe volume is large enough to cover the area under the coverslip
	4b. Air bubbles caught during Probe Mix application or mounting	4b. Avoid air bubbles. If observed, gently tap them away using forceps
5. Excessive background staining	5a. Inappropriate tissue fixation	5a Ensure that only formalin-fixed, paraffin-embedded tissue sections are used

Problem	Probable Cause	Suggested Action
	5b. Paraffin incompletely removed	5b. Follow the deparaffinization and rehydration procedures outlined in Section B.2
	5c. Stringent wash temperature too low	5c. Ensure that the stringent wash temperature is 65 (±2) °C
	5d. Prolonged exposure of hybridized section to strong light	5d. Avoid long microscopic examination and minimize exposure to strong light
6. Poor tissue morphology	6a. Incorrect Pepsin treatment	6a. Adhere to recommended Pepsin incubation times. See section B.3, step 2. Ensure that the Pepsin is handled at the correct temperature. See Section B.1
	6b. Incorrect pre-treatment conditions may result in unclear or cloudy appearance	6b. Ensure that the recommended pre-treatment temperature and time are used
	6c. Too long Pepsin treatment or very thin section thickness may cause ghost cells or donut cells to appear.	6c. Shorten the Pepsin incubation time. See section B.3, step 2. Ensure that the section thickness is 3-6 µm.

NOTE: If the problem cannot be attributed to any of the above causes, or if the suggested corrective action fails to resolve the problem, please call our Technical Services for further assistance.

Appendix 4 -Gastric

HER2 FISH pharmDx™ Kit, Code K5331

Protocol Checklist

Staining Run Log ID:_____

Date (Day 1) of the run: _____
HER2 FISH pharmDx™ Kit, K5331 Lot: _____
Specimen ID: _____
Equipment ID: _____
Date of dilution/expiration of the 1 x Wash Buffer (Vial 6 diluted 1:20):_____/_____

Tissue fixed in neutral buffered formalin	**Yes** □ **No** □

DAY 1

Step 1: Pre-Treatment	
Date of dilution/expiration of the Pre-Treatment Solution (Vial 1 diluted 1:20)	/
Measured temperature of Pre-Treatment Solution (95-99 °C) if water bath is used for heating	°C
Pre-treatment (10 minutes), and cooling (15 minutes)	
Wash in Wash Buffer (Vial 6 diluted 1:20) (2 x 3 minutes)	
Step 2: Pepsin	
Duration of Pepsin (Vial 2) treatment at 37 °C or	Minutes
Duration of Pepsin (Vial 2) treatment at room temperature	Minutes
Wash in Wash Buffer (Vial 6 diluted 1:20) (2 x 3 minutes)	
Dehydrate slides (3 x 2 minutes) in graded series of ethanol and air dry	
Step 3: *HER2*/CEN-17 Probe Mix	
Apply Probe Mix (Vial 3), coverslip and seal with Coverslip Sealant	
Measured denaturation temperature (82 ±2 °C)	°C
Denaturation for 5 minutes	
Measured hybridization temperature (45 ±2 °C)	°C
Hybridization overnight (protect from light)	Hours

DAY 2

Step 4: Stringent Wash	
Date of dilution/expiration of the Stringent Wash Buffer (Vial 4 diluted 1:20)	/
Measured temperature of Stringent Wash Buffer (65 ±2 °C)	°C
Stringent wash (10 minutes) after removing the coverslips	
Wash in Wash Buffer (Vial 6 diluted 1:20) (2 x 3 minutes)	
Dehydrate slides (3 x 2 minutes) in graded series of ethanol and air dry	
Step 5: Mounting	
Apply 15 µL of Fluorescence Mounting Medium (Vial 5) and coverslip	

Comments:_____

_____

Date and signature, Technician:_____

Appendix 5 - Gastric

HER2 FISH pharmDx™ Kit, Code K5331

Scoring Scheme

Date (Day 1) of the run: _____ Staining Run Log ID: _____

HER2 FISH pharmDx™ Kit, K5331 Lot: _____ Specimen ID: _____

Characterization of signal distribution in tissue:

Homogeneous ☐

Heterogeneous – Foca ☐ *or* *Heterogened* ☐ *– Mosaic:*

Count signals in 20 nuclei					
Nucleus No.	*HER2* score (red)	CEN-17 score (green)	Nucleus No.	*HER2* score (red)	CEN-17 score (green)
1			11		
2			12		
3			13		
4			14		
5			15		
6			16		
7			17		
8			18		
9			19		
10			20		
Total (1-10)			Total (11-20)		

For determination of the *HER2*/CEN-17 ratio, count the number of *HER2* signals and the number of CEN-17 signals in the same 20 nuclei and divide the total number of *HER2* signals by the total number of CEN-17 signals. If the *HER2*/CEN-17 ratio is borderline (1.8-2.2), count an additional 40 nuclei and recalculate the ratio for the 40 nuclei (refer to recount scoring scheme, Appendix 6).

A ratio at or near the cut-off (1.8-2.2) should be interpreted with caution (see counting guide).

	HER2	CEN-17	*HER2/CEN-17 ratio*
Total score (1-20)			

☐ Ratio < 2: *HER2* gene amplification was not observed

☐ Ratio > = 2: *HER2* gene amplification was observed

Date and signature, Technician: _____

Date and signature, Pathologist: _____

For scoring guidelines: see Interpretation of Staining.

Appendix 6 - Gastric

HER2 FISH pharmDx™ Kit, Code K5331

Recount Scoring Scheme

***HER2* FISH pharmDx™ Kit, K5331 lot:** _____ **Staining Run Log ID:** _____
Date (Day 1) of the run: _____ **Specimen ID:** _____

Signals in additional 40 nuclei (1-40)											
Nuclei no.	**Red** *HER2*	**Green** CEN-17	**Nuclei no.**	**Red** *HER2*	**Green** CEN-17	**Nuclei no.**	**Red** *HER2*	**Green** CEN-17	**Nuclei no.**	**Red** *HER2*	**Green** CEN-17
1			11			21			31		
2			12			22			32		
3			13			23			33		
4			14			24			34		
5			15			25			35		
6			16			26			36		
7			17			27			37		
8			18			28			38		
9			19			29			39		
10			20			30			40		
Total 1-10			Total 11-20			Total 21-30			Total 31-40		

For determination of the *HER2*/CEN-17 ratio, count the number of *HER2* signals and the number of CEN-17 signals in the same 40 nuclei and divide the total number of *HER2* signals by the total number of CEN-17 signals. **Report Total Score from the 1-40 nuclei in the table below.**

HER2 FISH	*HER2*	*CEN-17*	*HER2/CEN-17 ratio*
TOTAL SCORE (1-40)			

- ☐ Ratio < 2: *HER2* gene amplification was not observed
- ☐ Ratio > = 2: *HER2* gene amplification was observed

Date and signature, Technician: _____

Date and signature, Pathologist: _____

For scoring guidelines: see Interpretation of Staining.

Appendix 7 - Gastric

***HER2* FISH pharmDx™ Kit, Code K5331**

Fluorescence Microscope Specifications

Dako recommends the following equipment for use with the *HER2* FISH pharmDx™ Kit, K5331:

1. **Microscope type**
   - Epifluorescence microscope.
2. **Lamp**
   - 100 watt mercury lamp (keep record of burning time).
3. **Objectives**
   - For screening of the tissue, fluorescence dry 10X or fluorescence oil immersion 16X objectives are applicable.
   - For high power magnification and scoring of signals, only fluorescence oil immersion objectives, e.g. 100X are recommended.
4. **Filters**

Filters are individually designed for specific fluorochromes and must be chosen accordingly. Dako recommends the use of a specific DAPI filter in combination with a high quality Texas Red/FITC double filter.

- DAPI filter, e.g. Chroma filter # 31000.
- Texas Red/FITC double filter, e.g. Omega Optical filter # XF53 or Chroma filter # 51006.
- Texas Red and FITC single filters can be used for confirmation.

Fluorochrome	Excitation Wavelength	Emission Wavelength
FITC	495 nm	520 nm
Texas Red	596 nm	615 nm

Filters are specific to each microscope type and the use of appropriate filters is crucial for the interpretation. If you want detailed information, please contact your microscope provider or your Dako representative.

5. **Oil**
   - Non-fluorescing oil.

Precautions

- A 50 watt mercury lamp is not recommended.
- Rhodamine filters cannot be used.
- Triple filters are not recommended.

A non-optimized microscope may cause problems when reading the fluorescent signals. It is important that the light source has not expired and that it is properly aligned and focused.

Customers should monitor and follow the manufacturer's recommendations for the mercury lamp. The microscope should be maintained and the mercury lamp should be in alignment prior to interpreting results.

An effort should be made to expose the sample to as little of the excitation light as possible in order to minimize fading of the fluorescence.

We recommend that you discuss the set-up of your particular microscope with the manufacturer before starting the fluorescence in situ hybridization, or refer to the literature.

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Explanation of symbols

REF	Catalogue number	-20°C 8°C	Temperature limitation	LOT	Batch code		Toxic
IVD	In vitro diagnostic medical device		Keep away from sunlight (consult storage section)		Use by	F+	Extremely flammable
	Consult instructions for use	Σ	Contains sufficient for <n> tests		Manufacturer	N	Dangerous for the environment

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